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THE STRUCTURE AND FUNCTION OF THE ²⁴ LIVER OF *TEREDO*, THE SHIPWORM

By F. A. POTTS,

Fellow of Trinity Hall, Cambridge, and Lecturer in Zoology in the University.

(With Five Text-figures and Plates I and II.)

THE Teredinidae or Shipworms are a family of Lamellibranch molluscs, marine animals which rouse a particular interest because they are invariably associated with a terrestrial product, wood. Though they are not the only animal type which is so associated, they are by far the best known. In geological time the family was apparently already established in the Mesozoic, and later in the London Clay fragments of wood containing the burrows of a form assigned to this family are an abundant and characteristic fossil. In these distant ages the animal must have depended for its distribution on driftwood but after man appeared his wooden piers and his ships became a fresh source of refuge and food and in the case of the latter a particularly effective instrument of worldwide distribution.

The free-swimming larva settles on timber and immediately begins to burrow in it, the size of the animal and the burrows it produces increasing with almost incredible rapidity. In the comparatively cold waters of our own coast at Plymouth Orton (1914) found *Teredo navalis* in burrows 11 inches long in a raft only 31 weeks in the water. Sigerfoos (1908), in South Carolina, noted that in the allied form, *Xylotrya gouldii*, the burrows were already four inches long only five weeks after attachment and that the rate of growth was increasing rapidly. In tropical waters (Samoa) I have been able to observe that 29 days after a piece of wood is placed in seawater it contains shipworms which are liberating free-swimming larvae. Rapidity of growth and very early sexual maturity are, then, characteristics of the family.

It is now generally agreed that the burrow is excavated by the movement of the two valves of the shell which rasp away particles of wood. These, it is stated, are all swallowed and passed through the alimentary canal "as the only means of getting rid of them" (Sigerfoos). It is, however, recognised that the alimentary canal is highly modified in "adaptation to a bulky and highly innutritious diet" (Calman, 1919). The principal modification which is described is the presence of an enormous coecum of the stomach which is as long as the whole visceral mass and is always completely full of sawdust.

In spite, however, of these facts, writers on the shipworm are very dubious about its capability to make use of the wood as food. Sigerfoos (1908) speaks with peculiar authority owing to his very thorough investigation of the morphology and life-history of an American species (*Xylotrya gouldii*). "I am inclined to think," he says, "that they (i.e. the particles of wood) contribute something to the nutrition

of shipworms." But he evidently assigns the most important part to the diatoms and other floating organisms which are brought into the mantle cavity by the constant stream of water passing through the inhalent siphon. In lamellibranchs generally this is, of course, the usual source of food. These pabula are separated from the inhalent current by a ciliary mechanism, the details of which have been admirably worked out by Orton (1912), and sent forward to the mouth by a current flowing in the ventral groove of the gill lamellae. There is, thus, a strong presumption that this same method of feeding might be found to survive in the shipworms. Sigerfoos supposed that periods of boring alternate with periods of rest during which time the food collected by the gills is allowed to enter the mouth unmixed with wood, which may thus be better called feeding periods. So far as I have been able to gather this also represents the views of most biologists who have until lately thought about the matter. With regard to the possible digestion of the wood particles, Sigerfoos has two further suggestions to offer. Firstly, the coecum of the stomach has a large internal fold resembling the typhlosole of other invertebrates and this seems to him "so eminently constructed for absorption" that he thinks there must be some digestive action on the particles of wood within the coecum. Secondly, in the cavity of the lobules of part of the liver there are contained large quantities of wood and he therefore supposes that this portion of the liver may be specialised for the digestion of wood, possibly by secreting an enzyme for cellulose digestion which is conveyed to the coecum. In this second suggestion he was on right lines, as further research has shown, but he did not pursue them.

There are two ways in which the existence of such an enzyme may be demonstrated in *Teredo*. The first lies entirely within the province of the chemist and consists in the employment of methods such as making an extract from the tissues of the animal and demonstrating its chemical properties by test-tube reactions. But another method of approach, namely the direct observation of the cells of the liver and of their behaviour toward the cellulose of the wood also yields definite results.

From the biochemical side Harington, in a preliminary report (1921), offers evidence "in favour of the view that *Teredo* has in its liver an enzyme capable of producing glucose from some constituent of wood." The livers of several individuals were separated from the other tissues as carefully as possible, watery extracts of them made and the effect on cellulose, starch and fibrin estimated. Later still Dore and Miller (1923) employed another chemical method by making analyses of the wood in which the shipworm was boring and also of the castings which had passed through the alimentary canal. A comparison of these results indicated that wood loses 80 per cent. of its cellulose and a considerable part of its hemicelluloses in its passage through the digestive tract of *Teredo*. Taken together, then, these results place it almost beyond doubt that *Teredo* obtains carbohydrates from the wood it bores into and swallows.

When working over some sections of *Teredo* obtained in Samoa in 1920 I was struck by the curious appearance of the liver cells. Shortly afterwards I examined living material from Plymouth and to my great interest found the cells of a certain part of the liver packed with fragments of wood fibre like those found in the lumen of the organ. Since then I have pursued the subject at intervals and feel quite

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convinced that *Teredo* digests large quantities of wood and that, for the most part, the digestion is intracellular, a point which might indeed be expected from what is known of the digestion of other Lamellibranchs and, it may be said, of Molluscs in general.

The taxonomy of the Teredinidae is a subject which gives rise to the gravest differences of opinion and I do not therefore propose to say more than that the species of *Teredo* at Plymouth on which I have mostly worked is not at present accurately determined and that an identification would have to be accompanied by a description for which there is no room in the present paper. The phenomenon which is here treated is found throughout the genus, so far as my experience goes, and from the indications given in Sigerfoos' paper, also in *Xylotrya gouldi*. I have quoted largely from the anatomical description of the last-named genus because, so far as I can see, its anatomy is practically identical with that of *Teredo*.

My best thanks are due to Dr E. J. Allen, F.R.S., and the staff of the Marine Biological Laboratory at Plymouth and to Professor C. R. Harington, who kindly allowed me to use material from a raft which was kept there for his own researches.

THE LIVER OF THE MOLLUSCA.

The structure of the alimentary canal of the lamellibranch is extremely simple. The short oesophagus widens to form the stomach into which the large, richly branched liver glands open and from the stomach the intestine, which is usually of considerable length and often coiled, leads to the anus. The epithelium of the stomach may proliferate and become specialised to form derivatives of this organ. In the one case the liver is produced, in the other the groove or sac which forms the crystalline style. The rôle of the latter is still obscure, but, on the other hand, it is recognised that the liver, or hepatopancreas, as it is sometimes called, of the Mollusca and some other Invertebrata plays a much greater part in digestion than the similarly named organ of the Vertebrata. The view now generally taken is that the liver is the principal organ in these lower forms for the production of the digestive ferments and also for the absorption (or resorption) of the dissolved food as well as the storage place of reserve products like glycogen and fat.

One other well substantiated character of the molluscan liver which cannot but surprise those who derive their conception of a digestive gland from the Vertebrata is that the food materials ascend the liver duct and freely circulate in the lumen of the branches which may be seen to be full of solid particles. The duct is lined by ciliated epithelium which thus assists the ascent of the food into the lumen of the gland. This phenomenon is easily explained when it is realised that, at any rate, the final processes of digestion are often intracellular. It may be, as in the Coelenterata and *Helix*, that the secretion of a digestive juice causes a disintegration and semidigestion of the food in the cavity of the alimentary canal, but the smaller particles produced by this action are taken up phagocytically by the endodermal epithelium and completely digested. There is hardly, however, enough detailed evidence to warrant a general statement of this kind and it is more than likely that many exceptions exist. Possibly in one very specialised class, the Cephalopoda, intracellular digestion has been entirely eliminated.

THE ALIMENTARY CANAL AND LIVER OF THE TEREDINIDAE.

The accompanying figures, adapted from Sigerfoos, will serve better than any elaborate description to show the changes which occur in the course of growth from the newly attached Teredinid, with an alimentary canal of almost typical lamellibranch form, to the enormously elongated adult. In two points it may be mentioned here *Teredo* differs from other lamellibranchs throughout its development. These are the absence of labial palps (Calman is speaking in error when he states that *Teredo* possesses them and that they collect food) and the presence of a special diverticulum of the stomach, the coecum.

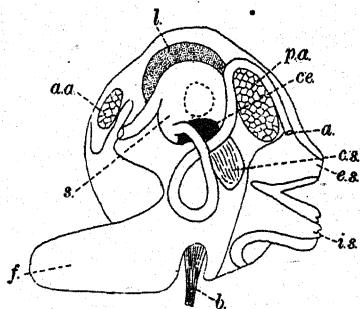


Fig. 1.

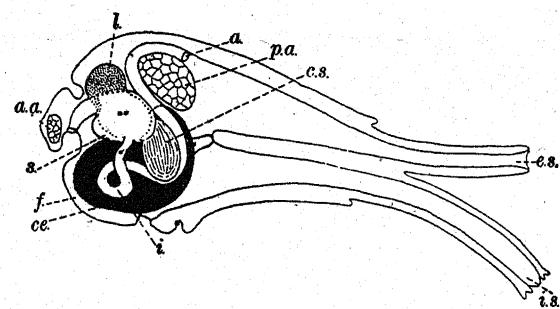


Fig. 2.

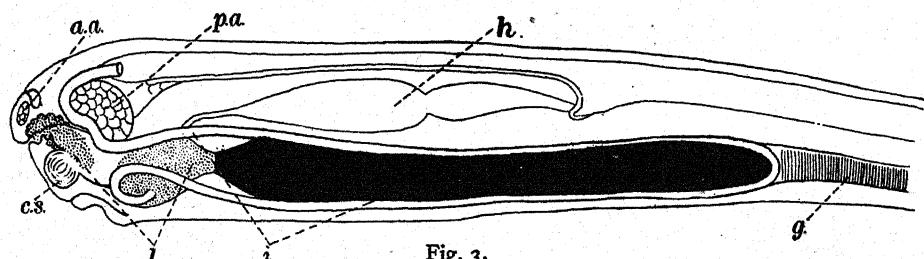


Fig. 3.

Text-fig. 1. *Xylotrya* which has just settled on wood. One lobe of the liver (the left) has been removed. Its opening into the stomach is indicated by a dotted circle.

In this and the two succeeding figures the liver is indicated by dots, the coecum of the stomach by black shading and the sac of the crystalline style by lines. The following lettering applies to all three figures:

a. anus, *a.a.* anterior adductor muscle, *p.a.* posterior adductor muscle, *e.s.* exhalent siphon, *i.s.* inhalent siphon, *c.s.* sac of crystalline style, *b.* byssus, *ce.* coecum of stomach, *f.* foot, *g.* gill lamina, *h.* heart, *i.* intestine, *l.* liver, *s.* stomach.

Text-fig. 2. *Xylotrya* after about 3 days' attachment.

Text-fig. 3. *Xylotrya*, adult. Left shell, mantle and gill removed in both. The liver is shown divided into two separate parts. At this stage, in *Teredo*, the posterior division of the liver really extends much farther back than is indicated here, sometimes covering half the length of the coecum.

(All figures slightly altered from Sigerfoos.)

In the newly attached form (Text-fig. 1) the stomach is rounded and from it on each side projects "a large almost spherical liver lobule." In the diagram the animal is shown with the left liver lobe removed, only a dotted line indicating its opening into the stomach. The intestine leaves the left side of the stomach

and just posterior to its point of origin is the coecum, a small hemispherical diverticulum. The posterior ventral part of the stomach is occupied by the opening of the sheath of the crystalline style, a large conical diverticulum which is median in position. This description is taken, with slight alterations in wording, from Sigerfoos and I will now quote him at length with regard to the changes occurring in later life:

As the larva develops into the shipworm, the size and relation of the parts of the alimentary canal change greatly. The oesophagus becomes in the adult very short in comparison with other parts. The stomach elongates posteriorly more and more, till in the adult, it projects far beyond the posterior adductor muscle and forms a long, more or less cylindrical tube.... Even before the ingestion of the wood begins, the coecum projects into the foot as a large hollow vesicle lined by clear ciliated cells: but as soon as wood is ingested it enlarges rapidly and soon forms the largest part of the digestive system.... As the visceral mass elongates the coecum is gradually drawn backwards till in the adult it forms a very long cylindrical tube stretching to the posterior end of the visceral mass... opening only at its anterior end into the stomach.

The above paragraph is illustrated by Text-figs. 2 and 3. It will be seen that though the length of the stomach increases greatly it is the extraordinary distension and elongation of the coecum which strikes the eye in this series of diagrams. It seems also that in its backward growth the coecum carries with it the intestine and is responsible for the corresponding extension of the latter organ.

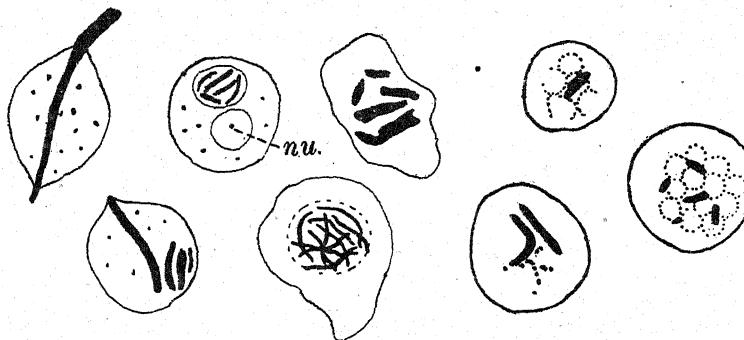
Sigerfoos goes on to describe the further development of the liver and I will make a further quotation from him:

The liver, composed of a single spherical lobule on either side of the stomach in the larva, soon divides into several lobules on either side. As growth takes place, the duct of the right half of the liver divides (in specimens 4-5 mm. long) and as the shipworm elongates, the posterior half of the right half of the liver passes backward, so that in the adult its duct opens into the posterior end of the stomach. These anterior and posterior portions of the liver are completely separated from each other, forming separate liver masses. The anterior remains in the foot and sends its duct to open into the lateral anterior portion of the stomach. There seems little doubt that it was this part which Frey and Leuckart observed and described as the salivary glands peculiar to shipworms. The posterior part of the liver is the larger of the two and opens by a very large duct into the ventral part of the stomach. It is differentiated into two portions, which in structure, and apparently in function, are quite distinct from each other though they open into the stomach by the same duct. The more elongated, slightly larger portion lies on the right side and in structure is like the anterior liver mass of shipworms and the whole liver in other forms of lamellibranchs. The second portion, lying more on the left side, is different in appearance. Its lobules are larger, with larger lumens and thinner walls, which are composed of flattened cells glandular in appearance. The presence of large quantities of woody materials in these larger, thin-walled lobules suggests that this portion of the liver may be specialised for the digestion of cellulose....

I have given this quotation in full to show that Sigerfoos clearly recognised two interesting facts about the liver of *Teredo*. The first is the separation of the liver into an anterior and a posterior region. There is a corresponding disjunction of the gill lamellae, as we shall see later. The second, which is far more important, is the division of the liver into two parts differing histologically.

On dissecting a fresh *Teredo* it is usually easy to distinguish, in the long compact posterior division of the liver lying to the left of the stomach, a brown and a white region (Pl. I, fig. 2). Each is composed of innumerable lobules. The first, which I will call here the *excretory* division of the liver, is usually dorsal and the second, the *digestive* division, is ventral, but often the relations are not so simply described. To represent the relations of the parts of the liver a diagram (Pl. I, fig. 1) of a transverse section is given here. In reality the liver duct branches on leaving the stomach and each division is much longer and narrower than is shown here. The duct is easily distinguished by the investment of cilia, external as well as internal. It contains great quantities of wood fibres. The walls of the duct are of moderate thickness and the cytoplasm contains many colourless granules.

The *digestive* portion of the liver consists of short rounded lobules. These are full of wood fibres and of a great number of free cells, varying in size, and usually containing numbers of wood fibres (Text-fig. 4). On pressing the coverslip the lobules



Text-fig. 4. Group of phagocytic cells from "digestive" part of liver showing the varying number and size of the wood fragments (black) taken up by such cells. Those to the left show also small granular inclusions, those to the right the vacuolisation of the protoplasm (indicated by dots). In the centre the wood fragments are shown enclosed in single enlarged vacuoles. *nu.* nucleus. $\times 1000$.

are ruptured and these cells escape. It may then be seen that they are constantly putting out slender pseudopodia, which extend, contract and coalesce very rapidly. Usually, however, this capacity for extension is lost when the cells contain a large number of wood fragments. Often a fibre is seized which is longer than the phagocyte itself. In the majority, however, the fragments are small and appear to have been corroded. Sometimes they are contained within a single large vacuole. In all cases the cells are uninucleate, the cytoplasm being clear and vacuolated. When a large number of wood fragments is contained in the cell, its outline is shrunken or crumpled.

The epithelium of these liver lobes is a single layer of cells, often with ill-defined boundaries or none at all. They form a very thin wall, with here and there a projection into the lumen of the lobule, where proliferation has taken place. Where the wall is thin it has an internal investment of long cilia, beating with a rather languid motion; and easily retractile, so that, when ordinary reagents are used for fixation of material, it is impossible to demonstrate them in sections. The projections

are not clothed with cilia but put out very slender pseudopodia (Pl. II, fig. 6), which constantly change their shape and resemble those of the free cells. They can be seen when fresh liver is teased out and compressed under a coverslip and observed with $\frac{1}{2}$ -inch objective. The transparency of the tissue allows satisfactory observation of the interior of the lobule. The oscillatory movement of wood fragments and free cells is seen to be due to the movement of the cilia of the border.

Not only does the liver epithelium project pseudopodia into the lumen but it takes up wood fragments into its cytoplasm. This is easily seen in living tissue and in sections (Pl. II, fig. 6). It is tempting to suppose that the free cells are formed from the phagocytic cells of the liver wall which proliferate, the internal portions being finally nipped off into the cavity. Such, indeed, is probably the case—the evidence of sections makes it very likely (Pl. II, fig. 7) but I have not seen it taking place in living tissue. Some of the free cells are very small with clear cytoplasm and no inclusions. Occasionally, however, even these are seen ingulflng wood particles.

That the wood taken up by these cells is partially digested by them is more than likely. It is, however, difficult to offer absolute proof. The process of digestion is so slow that though the same phagocyte is watched for protracted periods no marked change takes place in the outline of particular wood fragments. One minor piece of evidence is the occurrence of plentiful fat globules in the epithelium of the liver wall (Pl. I, fig. 4), which can be explained by supposing that the sugar derived from the digestion of the cellulose is in its turn converted into fat and stored in the liver cells themselves.

Lastly, it should be stated that Sigerfoos, without doubt, observed in *Xylotrya* the phenomenon I have described, for in his figure 58 B of Plate XIX he distinctly represents wood fibres within the cells of the liver, but makes no reference to the occurrence in the text.

In the *excretory* portion of the liver (Pl. II, fig. 8) the lobules are more numerous, and much slenderer. Their cavity also contains wood fragments and free cells. The epithelium of the lobule has immensely long cilia which are in constant motion and keep the contents of the cavity in circulation. But none of these cells are phagocytic—they can never be seen to put out pseudopodia nor do they ever contain fragments of wood. But both the free cells and the epithelial cells possess inclusions varying in size from minute rounded granules, clear but highly refracting, to enormous concretions occupying the whole cell and formed by the growth and coalescence of the former. They are yellow in colour, have no ascertainable structure and are resistent to most reagents, being practically insoluble in strong acids or alkalis. It is these which gives this region of the liver its characteristic colour and appearance.

Another feature of the liver, which is extraordinary and difficult to explain, is the presence of groups of ciliated cells (Pl. I, fig. 5), particularly in the part of the duct where it divides to form the lobules. These are sometimes single cells, sometimes rounded masses, in which on fixing and staining as many as a dozen nuclei can be recognised. In the latter case they are thickly covered with cilia—sometimes the covering is not complete. In histology they resemble the cells of the duct and as

actually this tissue is very much thickened at the end of the duct and extends into the lobules, it seems likely that the free ciliated cells are derived from it. What their further history and function may be I am at present unable to suggest.

I have compared the liver of *Teredo* with that of other lamellibranchs in which it was possible that similar phenomena might occur. *Xylophaga* is a form stated to be intermediate between the Teredinidae and the Pholadidae which occurs in wood, usually in twigs and branches, in shallow water at Plymouth. It makes very short burrows and the form of the animal is rounded not elongated. Though it has not undergone the extreme changes of shape of a typical Teredinid it has similar habits of life so far as my observations go. There is a stomach coecum entirely filled with minute wood fragments which also freely circulate in the liver ducts and lobules. The lobule cells are provided with long cilia which keep the contents of the cavity in constant motion. All the lobules, however, are of the same type as those present in the "excretory" portion of a Teredinid liver, the cells containing numerous inclusions, probably of an excretory nature. In no portion of the liver do phagocytic cells occur. In the colourless liver ducts the wood fragments are aggregated together, it sometimes seems, by a secretion from the cells of the duct, and I feel inclined to suggest that the liver "ducts" constitute the region where digestion mainly occurs.

I also examined the liver of *Pholas*, as another burrowing type, which is also probably related to the Teredinidae. Calman states that some species of the genus have occasionally been found in timber, but they usually burrow in rock. The sand formed by the rotatory action of the valves is not taken into the alimentary canal. The liver lobules are entirely of the "excretory" type, also resembling those described in *Teredo* in the strong internal ciliation.

It seems likely, then, that the possession of a region of the liver in which large free phagocytic cells are found is a peculiar character of the Teredinidae. It is associated with the great development of the capacity for digesting wood (which is already possessed by *Xylophaga* to some extent) and a very rapid rate of growth. When more is known about the life of *Xylophaga* and other forms where the habit of burrowing in wood is not so strongly developed, possibly more light will be shed on the problem of digestion in *Teredo*.

A curious feature in the above description is the occurrence of both cilia and pseudopodia in the cells of the same liver lobule. The cilia are highly retractile and the pseudopodia do not usually appear in sections but are very clear in the living tissue. Not only are they seen in the free phagocytes which come out of the cavity of the lobules when a piece of tissue is teased up but on examining a transparent lobule under pressure of a coverslip the phagocytic parts of the liver epithelium is seen, with ingested fragments of wood, and pseudopodia can be made out, with a $\frac{1}{2}$ " objective, projecting into the cavity. I think that the epithelium passes through phases and that ciliar retraction is followed by the putting out of pseudopodia and ingestion of wood. In such places there is also a multiplication of the nuclei accompanying assimilation, and separation of uninucleate phagocytic cells. That a reverse change occurs and the epithelium can return to the ciliated phase is less likely.

Though this phenomenon of phase change in cells has not been described, so far as I know, in the Mollusca it is not new in other phyla. Perhaps the best known case is in *Hydra*, first investigated by Nussbaum and Jickeli. To follow Miss Greenwood's description (1888), the larger vacuolate cells of the enteron possess amoeboid ends and retractile cilia. "In specimens examined in the fresh state (amoeboid movement) is exhibited by the formation of blunt hyaline projections which arise from the apex of each cell." One or two retractile cilia are also given off from each cell but "I do not believe that they coexist with the blunt amoeboid protuberances and I have not seen them in living teased specimens. It seems likely to me that all cilia are retracted during a digestive act." Other authors (Steche) have commented on the difficulty of observing the cilia in fixed material. Nussbaum earlier described the occurrence of ciliary action for some ten minutes after teasing a fresh *Hydra* and says that only later blunt pseudopodia are formed.

A rather different type of the alternation of ciliary and absorptive phases is described in *Lumbricus* by Miss Greenwood (1892). Here digestion is effected mainly by the secretions of unicellular glands occurring throughout the length of the typhlosole and corresponding region of the gut and absorption is carried out by the surrounding cells. Internally they have a hyaline layer which may be pierced by cilia. When, however, an earthworm is fed with fat these cells absorb it, and this layer is replaced by a striated or rodded border, while the active cilia are retracted.

INTRACELLULAR DIGESTION IN MOLLUSCA.

In the liver of *Mytilus* there are numerous ciliated ducts leading into lobules, the cells of which are distinguished from the cells of the duct, according to List (1902), by the lack of cilia and the fact that the cells are full of inclusions. These inclusions are granules of two kinds, colourless and coloured, the colour of the latter depending largely on the nature of the food. In neither case do they appear to be excretory but probably consist of protein substance associated with pigments derived from the food. With regard to the asserted absence of cilia, I would not like to support this apparent difference without extended search. They would be more difficult to detect in fresh material of *Mytilus* than in *Teredo* or *Pholas* and would not be seen in preserved material as a rule.

Besides the granule cells, *Mytilus* (and the Oyster) possess ferment cells. There is nothing which could be placed in this category in *Teredo*.

A very interesting and well-known experimental phenomenon has been described in the liver of *Mytilus*, *Anodon* and other forms. If a little Indian or Chinese ink is added to the sea-water which contains a few *Mytilus*, a suspension of ink particles is obtained which is drawn into the mantle cavity of the mollusc and then into the mouth and alimentary canal. It is possible in even so short a period as two hours (List) to demonstrate that the granule-cells contain ink particles. List says that these particles are taken in through the "Cuticular-saum" of the cell—a border of fibres at right angles to the cell surface—and find their way into the vacuoles of the cytoplasm. At first they adhere to the wall of the vacuole, but this gradually fills up until a solid ball is formed. After the experiment has lasted a

single day the liver appears quite black and almost every granule cell is full of such balls of ink. When a day or two more have passed these are thrust out again from the cell into the lumen of the lobule. Ingestion and rejection of ink particles goes on simultaneously.

A curious feature of List's account is his explanation of the manner in which the ink particles are taken into the cell through what is regarded as a permanent and definite cell organ the "Cuticular-saum." It would seem more likely that some form of pseudopodia cooperates in the ingestion of such solid bodies. I have made preliminary experiments with *Teredo* to compare with *Mytilus* and the other lamellibranchs and found to my surprise that the cells of the "excretory" part of the liver are actually capable of taking up such ink particles in the same way as the liver cells of *Mytilus*. They have no "Cuticular-saum" but a border of retractile cilia. I have not at any time seen amoeboid pseudopodia projecting from the free surface of the wall but as this occurs in the nutritive part of the liver it probably also occurs in the excretory part on occasion.

How far the absorption of ink particles may represent the natural stages of digestion is a point which naturally arises in the course of this enquiry. So many lamellibranchs live in a suspension of detrital particles and certainly obtain their food from the organic material amongst them that the experimental and natural conditions appear to be very close. The larger and heavier inorganic particles of the detritus would be separated out, the smaller and lighter organic material would be drifted by ciliary action to the spaces of the liver follicles and there engulfed in the cytoplasm of the cells, retained until all that is digestible is removed and the rest returned to the lumen of the organ. Such detrital particles are not, however, easily recognised in the liver cells of the lamellibranch so the proof of this possibility is lacking as yet. In *Helix* Jordan states a cytase secreted by the liver dissolves the cell walls of the plant food liberating the protoplasm with its granules which are carried into the liver follicles and ingested by the cells so that intracellular digestion follows the initial extracellular. A far clearer case, however, is that first described by von Bruel in *Hermaea*, a nudibranchiate gasteropod belonging to the Ascoglossa. The animal lives on the filaments of green Algae (often seeming to prefer members of the Siphoneae like *Codium* and *Bryopsis*). It is easy to see how the slender branch of a *Bryopsis* is sucked into the mouth and a slit made by the radula (which is reduced to a single row of teeth). Immediately the muscles of the pharynx contract, enlarging the cavity, and the fluid protoplasm of the alga flows through the slit and the mouth of the nudibranch into the alimentary canal of the latter. Not only the pharynx but the so-called "liver" diverticula are provided with muscle fibres, so that alternating contractions and expansions, not always simultaneous in the different parts, maintain a rapid circulation of the food during and after the meal. *Bryopsis* possesses very large and easily recognisable chloroplasts and a few minutes after *Hermaea* has commenced to feed they can be seen moving rapidly in the liver diverticula in the cerata (which here do not open to the exterior). In an hour or two the chloroplasts are all contained within the epithelium of the diverticula (Pl. II, fig. 9, ch.). Further than this I have not followed them myself, but von Bruel in his original account

describes their appearance as altering little by little as digestion progresses until all that remains, a small amount of reddish detritus, is expelled from the cells into the coecal lumen. From time to time a collection of such material is evacuated from the anus as is easily observed.

GLYCOGEN IN *TEREDO* AND OTHER FORMS.

While no quantitative estimates of the amount of glycogen have been made in *Teredo* there can be no doubt that the content is exceedingly high. If an individual, the tissues of which have been slightly torn in extracting it from wood, is kept in sea-water, the glycogen diffuses out rapidly and the sea-water becomes highly opalescent in appearance and if a few drops of iodine are added a deep port-wine colour is obtained. The principal place of storage is the mantle. A small piece of this examined under a low power of the microscope shows large highly refractive bodies which occupy the meshwork of the connective tissue, dissolving out when the tissue is macerated in water. They give the customary reactions of glycogen with iodine. In some specimens examined this year the mantle was very thick indeed with a corresponding increase in the amount of stored glycogen. A piece of such mantle placed in the mouth tastes very sweet.

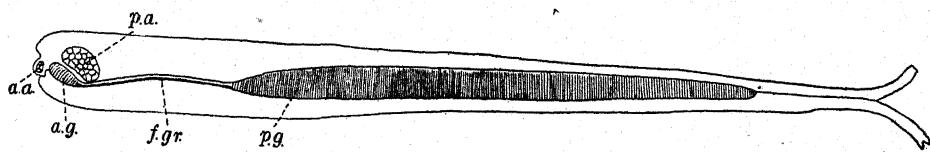
The mantle is, however, far from being the only organ in which glycogen storage takes place. A peculiarly interesting case was observed in March of last year (1922) when I examined specimens of small size (about 1 inch in length) and found them with embryos incubated in the gills. They were of comparatively large size and advanced development and were enveloped in a membranous tissue exceedingly rich in glycogen. It was difficult to resist the conclusion that the embryo thereby obtained nourishment from the mother.

The richness of the tissues of *Teredo* in glycogen seems to be directly due to the fact that the animal has the power of digesting cellulose. It is, however, interesting to note that in the oyster, which contains normally a very high proportion of glycogen, there is no indication that the animal digests cellulose at all. It lives on detritus mixed with diatoms and other small organisms in the composition of which cellulose and its derivatives play no important part. Some of the detritus is no doubt of vegetable origin, however, and it is just possible that this supplies carbohydrates to the mollusc.

Another animal which, constantly contains great quantities of glycogen is *Ascaris*. In this parasite the digestive function is almost completely in abeyance. The proteids and carbohydrates in the food of the host have been almost completely digested by the time they arrive in the intestine and so are ready for absorption by the cells of the midgut of the parasite without any further action by its own enzymes. Whether these cells exercise selection of carbohydrates or whether *Ascaris* manufactures its carbohydrate from the proteid absorbed is not known. It thus appears that the animals with particularly high glycogen content have very little in common, so far as their habits and diet are concerned.

A point of great interest in *Teredo* is the very great muscular activity which its manner of life must incur. The work performed by this form in boring, when the

length and size of the burrows is considered, would seem to be much greater in proportion than that done by any other lamellibranch. When removed from its burrow the shipworm does not show the movement of the shells so the method of boring remains a question of surmise. Slow but powerful contractions of the adductor muscles are required to explain the effects. Sigerfoos remarks that "all of the muscle fibres of both adductors are apparently striated, due to a more or less regular deposit of granular material on their surface." He goes on to compare the conditions with those in *Pecten* where one part of the posterior adductor is composed of non-striated muscle and tend "to keep the valves from separating too far" while the larger part, composed of striated fibres, causes very rapid adduction of the valves bringing about the swimming movements of the mollusc. I can find no structure in the muscle of *Teredo* at all comparable to the striation of the fibres in *Pecten*, nor do I feel that the kind of contraction in the two forms of muscle fibre is the same. But the adductor muscle is red in colour suggesting the presence of haemoglobin* and this, together with the granular interstitial material mentioned by Sigerfoos, is also found in vertebrate muscle fibres which carry out powerful



Text-fig. 5. *Xylotrya*, adult, to show anterior (a.g.) and posterior (p.g.) parts of gill lamina, f.gr. food groove, a.a. anterior adductor, p.a. posterior adductor. Left lobe of mantle removed.

contractions. If this muscular activity is carried out at the expense of glycogen an almost necessary connection may be traced between the curious digestive peculiarity of the animal, the work which it performs and the material on which it is done. The production of glycogen is, however, in excess of that required for the muscular contractions of a healthy *Teredo* and so it accumulates in the tissues. It must, however, be stated that iodine does not give the coloration with the muscle of *Teredo* which would indicate the presence of large quantities of glycogen.

THE POSSIBILITY OF OTHER SOURCES OF FOOD FOR *TEREDO*.

It has been mentioned before that many authors are disposed to regard the shipworm as living on plankton, either entirely or to a great extent. In investigating the truth of this statement two lines of investigation have been taken. Firstly, I endeavoured to find if such a food-collecting mechanism existed and functioned in *Teredo* as in other lamellibranchs, and, secondly, I examined the contents of the gut very carefully.

(1) The great elongation of *Teredo* or *Xylotrya* has affected the development of the gills very considerably. It must be stated first that the gill of the *Teredo* on each side only represents the internal half of the molluscan ctenidium, the external half never being developed. But the most significant variation in structure,

* This is strongly supported by a positive result in the benzidine-peroxidase reaction.

directly attributable to the change in shape of the mollusc, has been described by Sigerfoos as follows:

In a young shipworm a half centimetre in length there is a continuous series of seventy-five or more gill filaments stretching from the mouth region around the sides of the body and posterior to the visceral mass. Soon afterward the "filament" between the 10th and 11th (usually) gill slits broadens from before backward. This growth increases till, in large specimens of the adult the anterior eleven filaments (it may be rarely 10 or 12) are separated from the rest of the gill by a space of 10 cm. or more. They (*i.e.* the anterior filaments) retain the structure and doubtless the function of gill elements though in the adult they form a series of simple bars.

Sigerfoos points out that though the gill is thus divided into two by the elongation of the body yet the two parts are connected by a ciliated groove. This corresponds morphologically and functionally to the food groove found along the distal edge of the gill lamellae of other lamellibranchs. Also the epithelial development on the face of the lamellae is the same. The "lateral," "latero-frontal" and "frontal" cells, with their characteristic cilia, are present corresponding to those described by Orton in other molluscs, as determining the food-collecting and sorting currents.

Not only is the essential food-collecting mechanism apparently intact in spite of the changes in the structure of the gill, but it does actually work. If *Teredo* is removed from its burrow and kept in water in which Indian ink is suspended, then it is found that the particles are taken in by the ventral siphon and collected in the food grooves of the gills, and in spite of the absence of labial palps and the removal of the animal from its natural habitat ink is taken into the mouth. A good deal, however, is rejected and can be seen through the mantle, moving along ciliated paths on the mantle surface toward the exhalent siphon. But of that which passes through the alimentary canal a certain amount does find its way into the lumen of the liver lobules and is there ingested by the liver cells. A curious fact in the few successful experiments which I have made up to the present is that the carbon particles were taken up by the cells of the excretory portion of the liver and not by phagocytes. I hope to find out whether this is usually the case or not. At any rate it is certain that, though these cells are crowded with inclusions which seem to be excretory and though their internal boundary is normally ciliated they yet have the power of taking up particles like phagocytes.

But though these observations and experiments show that the collector apparatus of *Teredo* does function I think that the material obtained in this way is only of slight value in the nutrition of the animal, for the reasons given below.

(2) Definite remains of organisms or material (except wood) which appears to be of organic origin are strikingly rare in the alimentary canal and almost entirely confined to the intestine where occasional diatoms are found. Very often these diatoms have not been digested, for inside their flinty cases there is fresh-looking protoplasm. On some occasions when the shipworms were sickly and had ceased boring the intestine contained a great deal of inorganic material. But even here and

always the stomach, the coecum and the liver contains nothing but wood fragments. While working at Plymouth in the spring of this year and last I found many individuals in which the stomach and the coecum were empty or nearly so, showing that boring had been in suspense for some time. In spite, however, of the fact that the supply of wood was interrupted these organs contained no collection of material such as might have been made by the ciliary mechanisms of the gill, so that the absence of such material is not due to the operation of boring alone. Further investigation into the action of the collector and rejector mechanisms will be made and especially into the conditions under which, in laboratory experiments, comparatively large quantities of Indian ink can be taken up. I may mention here that when *Teredo* is kept in a thick culture of diatoms the intestine was largely filled with undigested individuals but I did not find that they penetrated the liver.

Lastly, I wish to refer to two other observations. In the summer of last year I made some experiments on the feeding of *Teredo* at the Tortugas Laboratory of the Carnegie Institution of Washington. I quote the following summary account from the Year Book of the Institution for 1922:

A floating plank was taken in tow and found to be infected by *Teredo*. A section weighing about 800 grams (containing about 70 shipworms) was cut off and kept in an aquarium supplied with filtered sea-water only. Every day at the same time the section of wood was changed to a new aquarium, the faeces discharged during the past 24 hours collected, and their dry weight estimated. The experiment was continued for 10 days, and the yield of wood from the burrows kept at a high level throughout. Eggs were laid by many individuals, and at the end of the experiment, when many shipworms were preserved, they were nearly all found to be sexually mature, while at the beginning comparatively few of those examined were at that stage.

While these experiments extended over only a short period, they tend to prove that the shipworm can live, perform its work normally, and reproduce without the possibility of using plankton as food.

I do not wish to lay too much stress on this experiment, of which I hope to publish a fuller account later, but I think the results support the evidence from other observations.

In 1920 I made a series of experiments on the rates of growth of sessile marine organisms, in the harbour of Pago Pago, American Samoa. Small rafts and boxes of wood were moored in various situations and became infected by two species of *Teredo*. Growth proceeded so rapidly that in 24 days after the rafts were put in the sea they contained shipworms which were producing free-swimming larvae. In a month's time the burrows had penetrated the wood in all directions. In most cases the rafts were taken out of the water after about a month. In the case of a box which was left in the sea for three months, when it came to be examined the sides and top were completely riddled by the burrows, which contained, however, no living animals but only shells and pallets. In the bottom, which was protected below by a layer of cement, the shipworms had only penetrated later and there they were still alive, rendering it improbable that the extinction of the animal in

other parts of the box was due to external conditions. So thick was the infection and so rapidly did the animals grow in these tropical seas that in less than three months all available wood had been excavated in the box and any further burrowing meant penetration into neighbouring tunnels. At this point the animals did not merely suspend boring and live on plankton or detritus, but they died. I must make it quite clear that we were not dealing with a necessarily short-lived tropical species of *Teredo* but that the supply of wood is a principal factor in limiting the life of the mollusc. In another part of the harbour, very stout timbers were sunk on the reef at a depth of eight fathoms nearly twelve months before our visit. When we pulled them up they were found to have been largely eaten by *Teredo* which was present alive, however, in the middle of the thickest balks, wherever there was still wood to bore.

COMPARISON OF TEREDO AND TERMITES.

Probably the case which can most profitably be compared with *Teredo* is that of the Termites. Where the food of these has been studied it consists largely of wood. In a special coecum of the alimentary canal there lives a remarkable protozoan fauna belonging to three families of the Mastigophora. Grassi found that almost all these protozoa might contain ingested fragments, particularly of wood, like those found in the lumen of the gut, and suspected that these micro-organisms might have some importance in the digestion of the insects. In 1910, Buscalioni and Comes investigated very thoroughly the problem of Termite nutrition. They used micro-chemical tests (aniline sulphate and phloroglucin) to determine the composition of the inclusions in the body of the protozoa and put beyond doubt the fact that they were really the remains of plant membranes, mostly lignin but also cellulose. They thought it possible that the latter were pieces of cell wall from which the layers of lignin had been digested leaving the cellulose. It may be pointed out, however, in passing that Dore and Miller's biochemical work on *Teredo* indicates the cellulose and hemicelluloses as by far the most easily digested carbohydrates of wood, but I may remark too that, nevertheless, microchemical methods used with the latter form have not in my own experiments given any clear indication of differential digestion in the ingested wood.

To return to the observations of the Italian authors; they found that, using iodine dissolved in potassium iodide solution, a coloration indicating the presence of glycogen in the protozoan body was given. This was not the case in all, and where present it differed in degree, in different species and in the two sexes of a species. In *Trichonympha agilis* and in others where the concentration of glycogen was marked, a special region near the nucleus was concerned with its storage. These results indicated the formation of glycogen by the protozoa as a result of the digestion of cellulose.

The presence of sugar derived from the glycogen was also sought for, using the method of Raspail, and positive results obtained, the presence of sugar diffused through the protoplasm being shown. Sudan III, was employed as an indicator for fat, and while it was not demonstrated in the protozoa, the intestinal epithelium

of the Termite contained large quantities of fat globules. It appears then that, while the protozoa are not capable of transforming the carbohydrates into fats, as individuals of the intestinal fauna die, the sugar diffuses out of their bodies and is absorbed by the endodermal epithelium of the insect where it is partly converted into fat. The authors regard the association of Termites and Mastigophora as a singularly clear case of "mutualistic" symbiosis. The protozoa have the advantage of living in a favourable culture medium and the termites are able to employ the sugars and glycogen manufactured by the protozoa—"questi abilissimi chimici degli alimenti,—questi veri cuochi che sanno ricavare da un materiale così poco digeribile qual è il legno secco un cibo tanto nutriente e saporito."

We are not dealing with the interaction of two widely different organisms in *Teredo*, which has to be its own cook. But the action of the phagocytic cells of the liver is wonderfully like that of the termitophilous protozoa. Just as it is supposed that the death of the protozoon sets free stores of food for the host, so it is possible that it is only by the disintegration of the phagocytic liver cells that the sugar they contain becomes available for the other tissues of the mollusc's body and is stored as glycogen in the mantle and elsewhere.

SUMMARY.

(1) The liver of the Teredinidae is shown to consist of three regions, the physiological relations of which have not been fully elucidated:

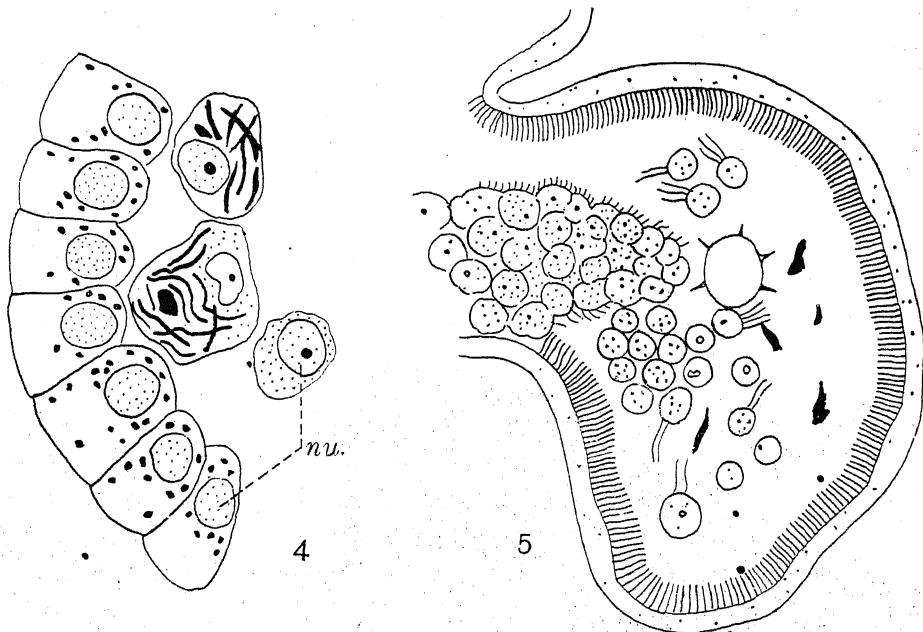
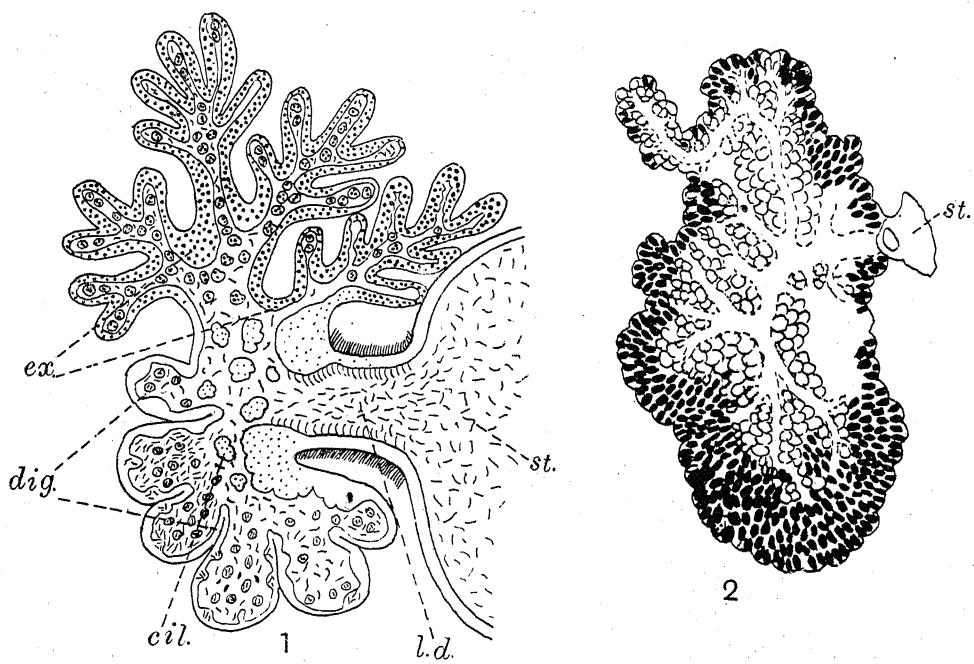
A. A "digestive" region in which the cells are phagocytic and nearly always contain minute fragments of wood. The phagocytic cells are mostly free in the lumen of the liver which also contains enormous quantities of wood. They are probably derived from the epithelium of the liver, which is also often phagocytic, containing wood fragments. Where this is not so the epithelium is ciliated internally.

B. In other lobules the liver wall is thickened locally and the lumen contains ciliated cells and cell aggregates, which are colourless, with small granular inclusions, and never contain wood.

C. An "excretory" region consisting of cells, also typically ciliated, containing large quantities of highly refringent, resistant granules whose chemical nature is not known but which are probably excretory. The lumen contains similar free cells.

(2) Glycogen has not been demonstrated in the liver but occurs in large quantities in the mantle and elsewhere. Fat globules are found in the epithelium of region A.

(3) The stomach, coecum and liver lumen contains no other recognisable organic material beside wood and only very occasional diatoms are found in the intestine. The gills of *Teredo*, though much modified, collect solid particles by ciliary action and forward them to the mouth. When kept in a suspension of Indian ink, the ink is found in the gut and the liver and even in the liver cells. In natural conditions material, which could have been so collected, is not recognised in those situations. Other evidence is given to indicate the shipworm's practical independence of plankton organisms.

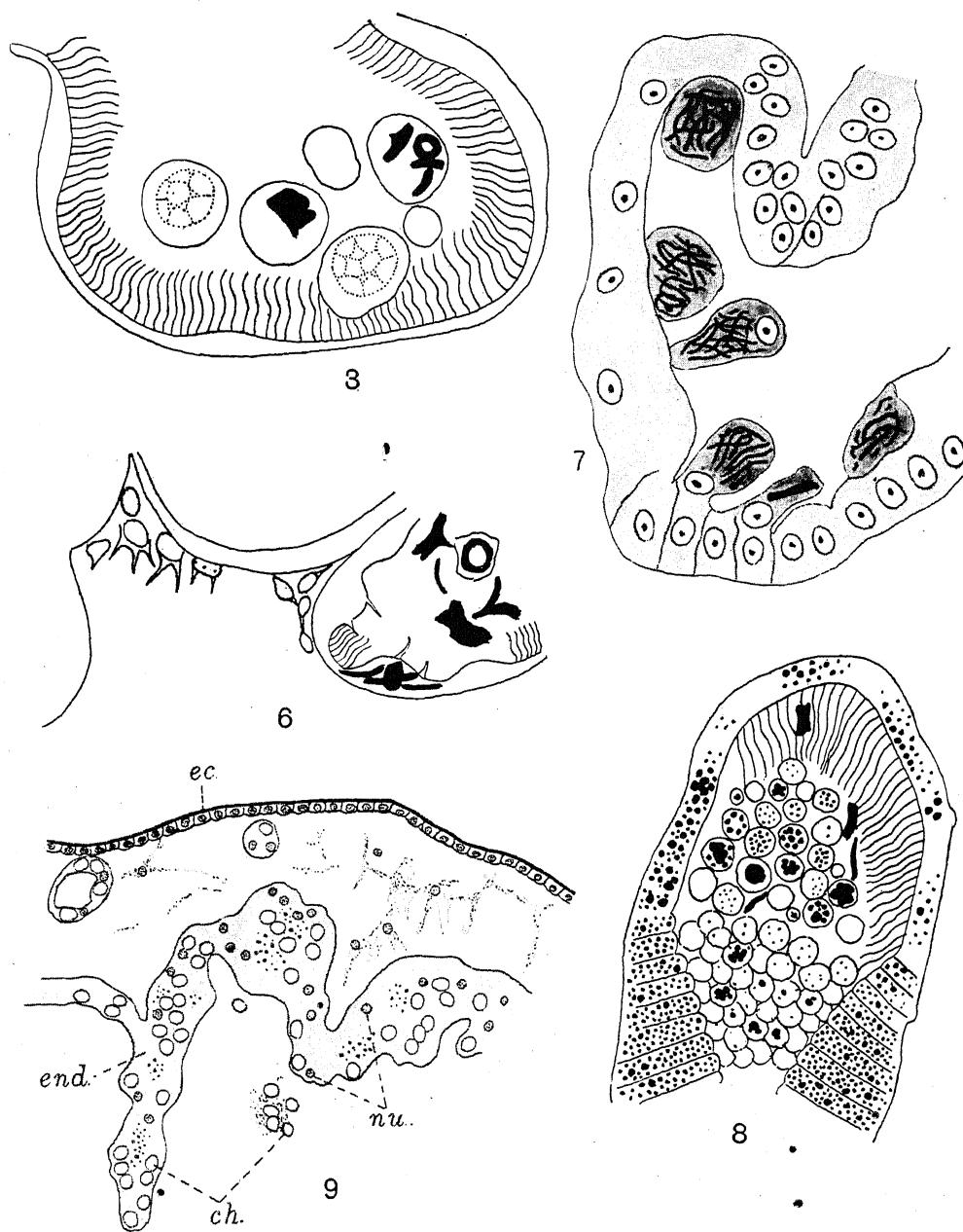
POTTS—THE STRUCTURE & FUNCTION OF LIVER OF *TEREDO* (pp. 1-17).

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POTTS—THE STRUCTURE & FUNCTION OF LIVER OF TEREDO (pp. 1-17).

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DESCRIPTION OF PLATES I AND II.

FIG. 1. Diagram illustrating the relation of the parts of the liver and stomach shown as in transverse section. *st.* stomach, *dig.* digestive and *ex.* excretory part of the liver, *l.d.* liver duct (very much shortened), *cil.* ciliated cells.

FIG. 2. View of face of liver to indicate the central position of the digestive part of the liver (white lobules) and peripheral position of the excretory lobules (black). The branching of the liver duct is also shown. *st.* torn membrane of stomach. $\times 8$.

FIG. 3. Lobule of liver examined alive under coverslip, showing the thin ciliated wall and the free cells both with and without included wood fragments. The latter cells show vacuolisation of the cytoplasm. $\times 1000$.

FIG. 4. Section of lobule of liver, Flemming and Iron Haematoxylin. Wall of liver composed of distinct cells with blackened fat globules. Phagocytes shown, two crammed with fragments of wood. *nu.* nuclei. $\times 1200$.

FIG. 5. Lobule of liver examined alive under coverslip, showing the ciliated wall and the thickened tissue at base which is giving rise to free ciliated cells without inclusions. A single phagocytic cell with short pseudopodia is seen. $\times 800$.

FIG. 6. Border of lobule examined alive under coverslip, showing ciliated wall raised locally into non-ciliated prominences, giving out pseudopodia and in one case containing many wood fibres. The lumen is full of irregular fragments of wood. One of them contains a bordered pit. On the outside of the lobules is a very characteristic investment of leucocytes with short processes. $\times 800$.

FIG. 7. Section through lobule (Flemming and Iron Haematoxylin), showing varying thickness of wall and phagocytic cells which here and there are in process of separation from the epithelium and are simultaneously taking up wood fragments. $\times 1000$.

FIG. 8. Tip of a liver lobule from excretory part of kidney showing the "excretory" granules (black), the lumen, enlarged terminally, and the epithelial cells with long cilia. Free cells, containing excretory granules of various sizes and a few wood fragments, occupy the lumen. None of the wood fragments are included in the cells. $\times 800$.

FIG. 9. Section through one of the cerata of an individual of *Hermaea dendritica* which was fed on the Green Alga, *Bryopsis*, an hour before being killed. Corrosive sublimate and Delafield's haematoxylin. *ec.* ectoderm, *end.* endodermal epithelium lining the "liver" diverticulum and containing *ch.* chloroplasts of *Bryopsis*, *nu.* nuclei. $\times 450$.

THE STRUCTURE AND LIFE-HISTORY OF *LIPOTROPHÄ* N.G., A NEW TYPE OF SCHIZO- GREGARINE, PARASITIC IN THE FAT BODY OF A DIPTEROUS LARVA (*SYSTENUS*)

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(From the Molteno Institute for Research in Parasitology, University of Cambridge.)

(With Two Text-figures and Plates III and IV.)

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I. INTRODUCTION.

THE present paper deals with the structure and life-history of two very closely allied Schizogregarines, which differ markedly from all the previously known forms and which are herein described respectively under the names of *Lipotropha macrospora* n.g., n.sp. and *Lipotropha microspora* n.sp. As their generic name shows, they live as parasites in the fat body of their hosts, *Systemus scholtzi* and *Systemus adpropinquans*. The first species, *L. macrospora*, was found in *Systemus* larvae from a wound in a horse-chestnut tree, standing on the Downing site near the Molteno Institute; the second species, *L. microspora*, was found in a single larva of *Systemus* from a wound in an elm tree standing on the Caius College playing ground at Newnham. The sappy wounds of these two trees have already yielded a very interesting Insect fauna, with a series of new Protists previously described.

II. HOSTS: *SYSTEMUS SCHOLTZI* LOEW AND *S. ADPROPINQUANS* LOEW.

The larvae of *Systemus*, hosts of these Schizogregarines, are carnivorous and prey upon various other Dipterous larvae, which live in the same medium. In the horse-chestnut tree they feed mainly on the larvae of *Dasyhelea obscura*, *Mycetobia*

pallipes, *Rhyphus fenestralis* and *Rhipidia ctenophora*; in the elm tree, in addition to the first three larvae, they feed on *Aulacogaster rufitarsis* and very probably on a mite, *Hericia hericia*. In both trees the larvae of *Systemus* often attack each other or are attacked and destroyed by the carnivorous larvae of an Anthomyiid, *Phaonia cincta*. When attacked, they often escape with only a small wound, which in healing leaves a black scar on the cuticle.

III. *LIPOTROPHA MACROSPORA*.

SITE AND INTENSITY OF INFECTION.

In the advanced and final stage of infection the larvae of the host are easily recognised with the naked eye by their peculiar milky appearance. To detect the larva in the early periods of infection, when most stages of the parasite are present, it is necessary to examine each larva under the microscope separately, slightly compressed. It is then seen that in some of the larvae the two longitudinal perivisceral bands of the fat body are abnormal in shape, very lobulated, or even torn to pieces. These larvae, which are very rare—representing only from 4 per cent. to 6 per cent. of the total number examined—are parasitised by *Lipotropha macrospora*. For the study of the parasite the best results have been obtained either by fixing portions of the fat body in Schaudinn's solution and staining them *in toto* in haemalum or iron-haematoxylin, or by making sections of the whole body of the larva fixed in Carnoy and staining them in iron-haematoxylin and eosin or in Mann's methyl blue-eosin solution.

A small portion of the fat body of *Systemus* larva stained *in toto* is represented in Pl. III, fig. 1. It gives only an approximative idea of the intensity of infection, as a large number of parasites superposed to each other have been purposely omitted and only those lying in or near the same horizontal plane have been represented. In many other microscopic fields of the same fat body the parasites were crowded to such an extent that no attempt could be made to represent them in a drawing. In these places they formed a solid mass, replacing completely all the elements of the cells of the fat body except their nuclei, which although pressed between the various stages of the parasites, retained their normal size and structure.

SCHIZOGONY.

The youngest stages of the parasite found in the fat body of the host are small elongated bodies 4μ to 6μ long and 0.9μ to 1.5μ across their widest portion. They (Pl. III, fig. 6 a) show a very small nucleus, of which there is clearly seen only the large karyosome, surrounded by a clear zone representing the remaining portion of the nucleus. The anterior end of the parasite at this stage is rounded while the posterior is pointed. These bodies, which probably represent the young merozoites, become spherical and 2μ to 2.5μ in diameter (Pl. III, fig. 1 a). In this stage they grow and before they reach 5μ their nuclei divide mitotically many times, giving rise to a great number of small nuclei. The small size of the parasite and especially of the spindle during the division makes it impossible to give any interesting detail con-

cerning this process. Simultaneously with the nuclear division the whole body of parasite grows (Pl. III, figs. 2 and 3) and reaches the size of 12μ in diameter. The nuclei become very numerous and evenly distributed in the peripheral layer of the parasite. Surrounded by a small quantity of protoplasm, they protrude from the surface of the body and give rise to as many small buds as there were nuclei. These buds grow in size, become elongated and the schizont is gradually transformed into a cluster of merozoites, grouped round the central residual mass of protoplasm (Pl. III, fig. 4). The schizont at this stage reaches 22μ in diameter and soon breaks up into numerous merozoites 4μ to 6μ long (Pl. III, figs. 5 and 6). These merozoites become spherical, growing into schizonts of the second generation, and this process of schizogonic multiplication goes on for a certain period of time, during which the fat body of the host becomes heavily invaded with the parasites.

SPOROGONY.

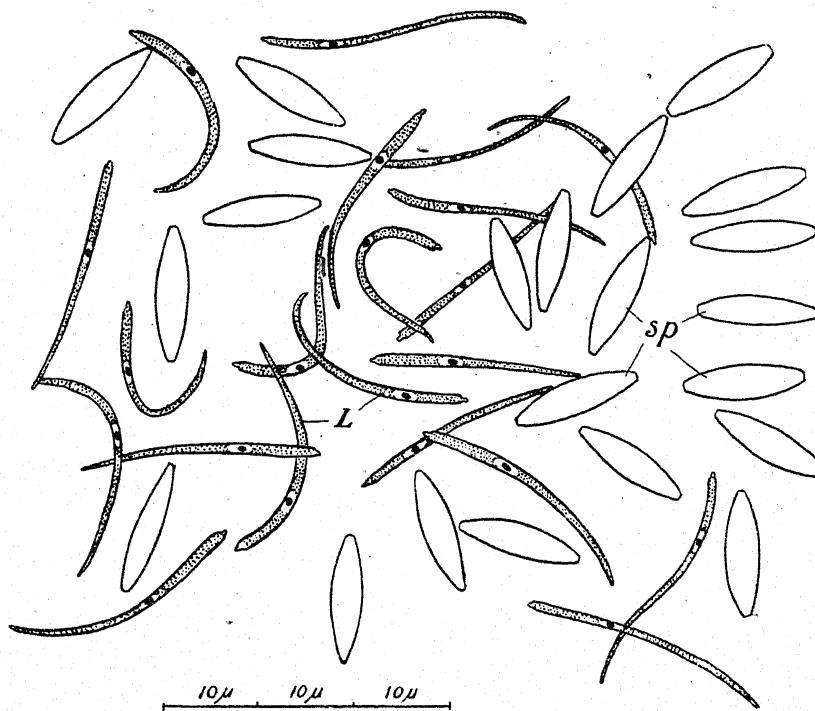
While the schizogonic multiplication is still in progress, some of the merozoites become spherical and, instead of giving rise to a new generation of schizonts, grow in size without any nuclear division, gradually forming the generation of gametocytes (Pl. III, fig. 1, c). A full-grown gametocyte is a more or less spherical body 10μ to 12μ in diameter, with vacuolar protoplasm containing a few scattered chromatic granules and a vesicular nucleus (2.5μ). The latter is almost devoid of peripheral chromatin but is provided with a karyosome (1μ) (Pl. III, fig. 1, e). The gametocytes associate in pairs, become hemispherical, contract their bodies and secrete a very thin transparent cyst, almost spherical in shape and about 10μ in diameter (fig. 1, f). The nucleus of each of the two encysted gametocytes undergoes four successive mitotic divisions, giving rise to 16 nuclei of gametes, which are formed by a process common to all Gregarines (g). The gametes of both gametocytes, similar in structure (isogamy), unite in pairs and form 16 spherical sporoblasts of about 3.5μ (h). Their protoplasm is vacuolar and very basophile; the nucleus, being very rich in chromatin, does not yet show the formation of the karyosome. These sporoblasts grow in size, become elongated and gradually give rise to sporocysts (i and k). The latter are truncated at both ends, 13.5μ long and 3μ across the middle, and provided with a very thin transparent wall. The nucleus of the sporocyst divides three times and gives rise to the nuclei of eight sporozoites. The longitudinal sections of ripe sporocysts show also two small basophile granules, one near each pole of the spore (Pl. III, fig. 13). During the process of spore-ripening the cyst becomes distended, reaches 14.5μ to 15μ in diameter and its thin wall frequently bursts, letting the spores escape into the free spaces left in the fat body of the host (l).

THE FINAL STAGE OF INFECTION.

When the cysts and spores of the parasite have invaded more or less completely the host's fat body, the latter breaks up into pieces of various sizes, which become loose in the perivisceral fluid of the larva. These pieces are seen passively moving from one segment into another and, being often squeezed between the segments

and various organs, finally disintegrate into small particles. This process goes on until no trace of the fat body can be detected and the parasites in every stage are all liberated in the blood plasma of the host. The fragile cysts of the parasite burst in their turn, setting free the spores, which occupy the body-cavity of the larva in great numbers.

Fresh cysts are no longer formed, although the gametocytes in various stages are still present in the blood. This is probably due to the constant energetic movement of the fluid medium where they are now found. The schizonts, on the other



Text-fig. 1. Smear of the perivisceral fluid obtained by pricking a *Systemus* larva during the last stage of infection, showing *sp.* numerous spores and *L.* active gregarine-like stages of *Lipotropha macrospora*. Smear fixed in Schaudinn and stained in iron-haematoxylin. Stain did not penetrate into the spores.

hand, undergo further development, the ultimate stage of which differs from that occurring in the fat body. The young merozoites, which in the fat body became spherical and gave rise to a new generation of schizonts or gametocytes, being now free in the blood, grow in length and are gradually transformed into elongated organisms, 28μ long but only 1μ to 1.2μ in diameter. Examined *in vivo*, in a droplet of the host's blood, they may be seen bending and curving their bodies in various forms, such as those of a circle, *C*, *S*, or *U*. The anterior portion is constantly moving to right and left, then suddenly the whole body darts forward in a straight line, but soon stops and again after a short interval moves forward as before. They often move in groups of two, three or four, touching one another laterally, but never forming a real chain. On smears fixed and stained these organisms show anteriorly

a small knob-like projection (a kind of epimerite) and, near the middle of the body, a nucleus with a large karyosome (Text-fig. 1, *L* and Pl. III, fig. 6). These elongated, gregarine-like, active forms are produced in enormous numbers and finally, with the spores previously described, fill the whole body-cavity of the host. At this stage a parasitised larva can be easily recognised with the naked eye by its milky opaque appearance. By pricking such a larva with a fine needle there gushes out from the wound a milky fluid, which, when examined under the microscope, is seen to be composed of a very thick suspension of spores and elongated merozoites.

REACTION OF THE HOST.

An infected larva can never get rid of the parasites which multiply in its fat body until the latter is completely destroyed. The imaginal discs, being now devoid of all reserve substance, are arrested in their development and the larva never passes into the pupal stage, though it remains active and feeds upon other larvae living in the same medium. During the last period of infection the host begins to show a characteristic reaction against the parasite. The leucocytes of the larva are now seen taking up the parasites in the various stages as well as the remains of the cells of the fat body. The parasite however does not suffer much from this attack, especially the spores which always remain of normal shape and structure (Pl. III, figs. 7 to 11). The leucocytes, on the other hand, after having taken up a great number of parasites, degenerate and break up into small particles. It is interesting to note that active merozoites are very seldom found inside a leucocyte. Among several hundreds of leucocytes filled with various stages of *Lipotropha* only two have been found to contain more or less degenerated elongated merozoites (Pl. III, fig. 10).

MODE OF INFECTION.

Attempts to infect healthy *Systemus* larvae artificially, by feeding to them the spores of *Lipotropha*, have failed. It is possible however that the spores used in these experiments were not sufficiently ripe. In nature the spores of *Lipotropha*, after being liberated from the dead and disintegrated body of the host and scattered in the surrounding medium, are usually swallowed by various saprophagous larvae with their food. The spores reach the uninfected larvae of *Systemus* when the latter feed upon larvae carrying these spores, or when a healthy *Systemus* larva preys directly upon a parasitised one. Cannibalism being a common occurrence among these larvae is probably the cause of the cases of very heavy infection. From what we know about Gregarines in general, it may be presumed that the ripe spores of the parasite, on reaching the midgut of *Systemus*, open and let the sporozoites escape. The latter then penetrate the walls of the gut into the body-cavity and pass thence into the cells of the fat body.

Occurrence of Lipotropha macrospora in the larvae of Rhipidia ctenophora.

The wound or the decomposed wood pulp of a horse-chestnut tree, as has been already mentioned above, often harbours the larvae of a Tipulid fly: *Rhipidia ctenophora*. These larvae, before they reach maturity, are always transparent and

all the contents of their body-cavity can be thoroughly investigated without damage. Having had occasion to examine them by transparency for the study of an intestinal parasite, the writer has noticed on many occasions a number of elongated, gregarine-like organisms floating in the perivisceral fluid. Examined in a drop of blood, taken from such a larva by pricking it with a fine needle, the organisms show exactly the same structure and move in the same manner as the active elongated merozoites of *Lipotropha*, with which we have been dealing. They are only of a slightly larger size, being 34μ long and 1.4μ to 1.8μ across the widest portion, and show also a special affinity for the fat body of the larva where they may be seen, either fixed to the surface of the fat cells, or rolled in a spiral or circle inside the protoplasm (Pl. IV, fig. 2, L). This stage seems, however, to be the final one in their life-history, since examination of the fat body and other organs in numerous infected larvae did not reveal any further stage in the development. It is most probable that the parasites ultimately degenerate and disappear before the larva passes into the pupal stage. This case of accidental parasitism, within the body of an unsuitable host, will be discussed later in dealing with a similar case, which appears to occur in *Lipotropha microspora*.

IV. *LIPOTROPHA MICROSPORA*.

GENERAL CHARACTERS.

Among a lot of about 25 larvae of *Systenus adpropinquans*, collected from the wound of an elm tree standing on the Caius College playing ground, one larva was found to have an abnormal-looking fat body. This larva was fixed in Carnoy and some sections of it were stained in iron-haematoxylin and eosin or orange, the others in Mann's methyl-blue eosin solution. The study of these sections revealed the fact that the whole fat body of the larva was heavily infested with various stages of a Schizogregarine which bears much resemblance to that of *Lipotropha macrospora*. As Fig. 1 (Pl. IV) shows, the parasite has almost completely replaced the fat body of the host, leaving only patches of it and the nuclei (N) untouched. In one field of view of high magnification (oil immersion $\frac{1}{2}$ and compens. oc. 6) practically the whole life-history of the parasite can be seen. The latter has always a tendency to multiply around the fat droplets, which, being dissolved by Carnoy's fluid, are shown in the figure as white empty circular spaces. Its resemblance to *L. macrospora* is so pronounced that it will be quite sufficient for description to dwell only upon its most important and distinctive characters. It is proposed to name this parasite *Lipotropha microspora* n.sp. and its main characters are the following:

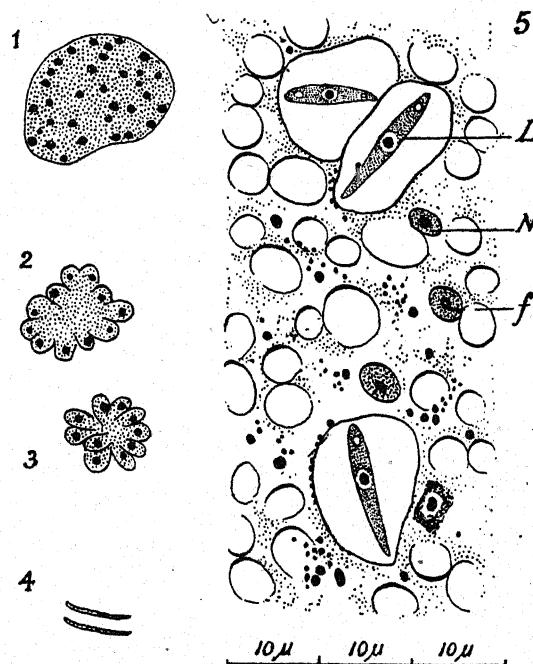
- (1) The schizonts of this parasite form rather short and stout merozoites; which surround the fat droplets, forming around them two, three or more concentric layers (Text-fig. 2 and Pl. IV, fig. 1, b).
- (2) The active elongated merozoites have not yet been found, but this may be explained by the fact that the fat body still contained some portions available for schizogonic multiplication and that the parasite was not yet liberated into the perivisceral body fluid.

(3) The gametocytes and the cysts are of smaller size than those of the previous species, reaching between 8μ and 9μ in diameter.

(4) The sporocysts are also of smaller size, being 8μ long and 3μ across their middle portion. They further differ from those of *L. macrospora* by being spindle-shaped and provided with a much thicker wall.

(5) Groups of very small elongated bodies 7μ by 0.5μ , found around the fat droplets, which could be regarded only as sporozoites of recent invasion, show that the sporozoites from the gut directly invade the fat body without passing through a preliminary stage in the body fluid (Text-fig. 2, 4).

These characters seem quite sufficient to separate this species from the previously described *L. macrospora*.



Text-fig. 2. *Lipotropha microspora*: 1. section of a large schizont; 2. and 3. sections of schizonts, showing the formation of merozoites; 4. two small sporozoites found around the fat globules; 5. piece of fat body of *Dasyhelea obscura* larva showing (L.) parasites, probably *Lipotropha microspora*. N. nuclei of the fat body; f. fat droplets.

Probable occurrence of *L. microspora* in the larva of *Dasyhelea obscura*.

The commonest larvae met with in the wounds of elm trees are those of a Ceratopogonine fly, *Dasyhelea obscura* Winnertz. A great number of these larvae have been examined for the study of various parasites but in only one was a certain organism found, for which for a long time no place could be found in any known group of Protists. The body of this organism is elongated, 14μ long and 2μ across the widest anterior portion; its anterior end is rounded, while the posterior is pointed. The protoplasm is uniform, its nucleus, 2μ in diameter, is poor in peri-

pheral chromatin but provided with a large karyosome. A small circular vacuole is present near the anterior end of the body. This organism is found only in the fat body of the larva, and the cells which are inhabited by it show no protoplasm in smears fixed in Carnoy. In these smears the parasites are invariably surrounded by a clear empty circular space, as if a whole cell had been previously occupied by one large drop of fat, which became dissolved during the subsequent histological manipulations (Text-fig. 2, 5 and Pl. IV, fig. 3, L). This parasite, which does not show any other stage of development, is undoubtedly of the same nature as the organism found in the larva of *Rhipidia ctenophora*. It is then quite possible that in this case, just as in that of *Rhipidia*, the parasite belongs to the corresponding species of *Lipotropha* which, having accidentally penetrated into the wrong host, has accomplished only a part of its life-cycle.

This partial parasitism of the two corresponding species of *Lipotropha* in two accidental hosts, *Rhipidia ctenophora* and *Dasyhelea obscura*, gives some indication as to how the parasites begin to adapt themselves to a new host. It is obvious that the spores of *Lipotropha*, being scattered in a very limited area, such as the wound of a tree, have a great chance of being swallowed by all kinds of saprophagous larvae living in the same medium. In most cases these spores pass unchanged through the alimentary canal of the larvae, in others they may be partially digested and destroyed; but there are, nevertheless, a few rare cases in which the spores, under the influence of the gastric fluid, open and the small sporozoites bore through the walls of the gut to reach the perivisceral body-cavity of the larvae. In the case of *L. macrospora*, they remain for a long time in the blood of *Rhipidia*, where they grow and become even larger than the active merozoites found during the last period of infection in *Systemus* larvae.

They show however some difficulty in penetrating the fat body of *Rhipidia* and, even when they reach it, do not seem to feed. In the case of the infection of *Dasyhelea* larvae by the supposed *L. microspora* the latter actually invades the fat body and seems to feed upon it. Although the development of the parasites stops at different periods, the interesting fact remains that in both cases they have accomplished several important and complicated portions of their life-cycle, namely, hatching of sporozoites in the lumen of the gut, boring through the walls of the gut, the period of life in the perivisceral fluid and invasion of the fat body.

V. GENERAL CHARACTERS OF THE GENUS *LIPOTROPHA*.

Lipotropha is a typical Schizogregarine, the main characters of which are the following:

- (1) During the whole of its life-cycle, schizogony and sporogony, the parasite lives within the fat body cells, *i.e.* the parasite is intracellular and feeds upon fat.
- (2) In its vegetative stage, the stage of copula and cyst, *Lipotropha* is the smallest organism known among the Gregarines, the gametocytes being only 8μ to 12μ and the freshly-formed cysts 10μ to 14μ in diameter.
- (3) The merozoites are small and numerous.
- (4) Cyst contains 16 sporocysts.

(5) Sporocyst comprises eight sporozoites.

(6) The stage of the active trophozoite is either completely suppressed (probably in *L. microspora*) or is reduced to a short period of life in the perivisceral fluid of the host (*L. macrospora*). At this stage they are 28μ long, 1μ to 1.5μ wide and provided with a very small knob-like epimerite.

Two species are described herein:

(1) *Lipotropha macrospora* n.sp., a parasite of larger size with spores 13.5μ by 3μ , truncated at both ends and provided with a thin wall. Living normally in the fat body of the larvae of *Systemus scholtzi* Loew and *Systemus adpropinquans* Loew, it also occurs accidentally in the blood and fat body of the larvae of *Rhipidia ctenophora* Loew. The hosts inhabit the wound of a horse-chestnut tree on the Downing site, Cambridge.

(2) *Lipotropha microspora* n.sp., a parasite of smaller size; merozoites short and tapering; spores spindle-shaped 8μ long and 3μ across the middle; wall of sporocyst thick. It occurs in the fat body of *Systemus adpropinquans* Loew and, probably accidentally, in the fat body of the larva of *Dasyhelea obscura* Winnertz. The hosts live in the wound of an elm tree on the Caius College playing ground, Newnham, Cambridge.

VI. SYSTEMATIC POSITION OF *LIPOTROPHA*.

As to the systematic position of the genus *Lipotropha* among the Schizogregarines, we cannot yet make any definite statement. All we can do is to mention briefly a few characters which it has in common with other known genera in this sub-order. Such are:

(1) The intracellular mode of life (schizogony and sporogony) of *Lipotropha*, which is known to exist in the genus *Spirocystis*.

(2) The number of spores in the cysts (16) which is just intermediate between that of *Caulleryella* (8) and *Schizocystis* (about 30).

(3) The intracellular schizogony which recalls that of *Selenidium*, *Mero-gregarina* and *Menzbieria*.

(4) Small size of gametocytes and cysts resembling those of *Ophryocystis*.

(5) Number of sporozoites in the spores (8) which is the same as in *Ophryocystis*, *Schizocystis* and *Caulleryella*.

(6) Mobile elongated merozoites of *L. macrospora* which bear some resemblance to vermiform trophozoites of *Selenococcidium*.

If we were to follow the tradition now established for the sub-order of Schizogregarinariae, we should have to introduce a new family of Lipotrophidae, since for every new genus described in this sub-order a new family has been created. This appears however to be completely superfluous, as it does not help to bring into this group any natural order. The attempt of Léger and Duboscq (1908) to divide it according to the number of spores in the cyst was perhaps justified when only a few forms were known, but it must now be abandoned, since it cannot be applied to new forms recently described. In fact, we have already species with 1, 8, 16 and more spores, and, as the difference between 8 or 16 and many is as

important as the difference between the same numbers and 1, we cannot divide all the species only into monospora and polyspora. Moreover the cysts with 1 spore occur in forms like *Spirocystis* and *Ophryocystis*, which are obviously very distinct and could not possibly belong to the same group.

A similar objection must be raised to the classification proposed by Fantham (1908), who divided the Schizogregarines into the Ectoschiza and Endoschiza, the first having an extracellular and the second an intracellular schizogony. This system of classification, which seemed satisfactory in 1908, cannot be applied for genera like *Cauleryella*, the schizonts of which are partly intracellular, or to the recently-discovered *Menzbieria*, which possess two kinds of schizonts, extra- and intracellular.

Moreover, we do not yet know whether the schizogony in this group is a primitive or only a secondarily-acquired character. In the light of the first supposition, which is supported by Léger (1910), the sub-order of Schizogregarinariae would be homogeneous and probably monophyletic in origin. If we accept the second supposition, which is strongly supported by Mesnil (1899), the group of Schizogregarines will have to be considered as heterogeneous and artificial and the various genera it comprises will sooner or later find their place in various families of Eugregarines from which they have been derived.

At present neither of these two suppositions is sufficiently supported by facts and it is therefore advisable to defer all new attempts at classification of the group to the time when further new forms have been described.

In the present state of our knowledge we must consider the group of Schizogregarines composed of the nine following genera¹:

Genera	No. of species	Hosts	Authors describing life-cycle
<i>Ophryocystis</i> A. Schneider	9	Coleoptera (Malpig. tubes)	Schneider, Léger (1907)
<i>Schizocystis</i> Léger	2	Diptera (larvae, gut)	Léger (1900, 1910), Keilin (1923)
<i>Cauleryella</i> Keilin	4	Diptera (larvae, gut)	Keilin (1914), Hesse (1918), Bresslau and Buschkiel (1919)
<i>Lipotropha</i> Keilin	2	Diptera (larvae, fat body)	Keilin—see this paper
<i>Menzbieria</i> Bogoyavlensky	1	Acarina (Hydrachna, gut)	Bogoyavlensky (1922)
<i>Porospora</i> A. Schneider	6	(Schizogony in Crustacea Decapoda)	Léger and Duboscq (1913 a, 1913 b, 1915 b)
<i>Spirocystis</i> Lég. and Dub.	1	Sporogony in Mollusca	Léger and Duboscq (1914, 1915)
<i>Selenidium</i> Giard	several	Lamellibranchiata Oligochaeta (various tissues)	Giard (1884), Caulery and Mesnil (1898, 1899), Brasil (1907, 1909), Fantham (1908—this paper contains almost complete bibliography and a list of species with their hosts), Léger and Duboscq (1917)
<i>Merogregarina</i> Porter	1	Polychaeta, Gephyrea Ascidia	Porter (1909)

¹ The forms like *Eleutheroschizon* Brasil (1906) and *Siedleckia* Caulery and Mesnil (1899) cannot be included among the Schizogregarines until their sporogonic cycle has been discovered. The genus *Selenococcidium* Léger and Duboscq (1910), parasitic in the intestine of the lobster, has much more affinity with the Coccidia than with the Gregarines and should be placed among the former, as has been held by Léger and Duboscq (1910).

The foregoing list shows that the greatest variety of forms of Schizogregarines has been found in Arthropods and especially in Insects. Among the latter Dipterous larvae alone have yielded three genera. Now, considering how very little these larvae have been examined by Protistologists, it is evident that a very wide field for discoveries of new forms of Schizogregarines and many other Protists is still awaiting investigators in this and other orders of Insects.

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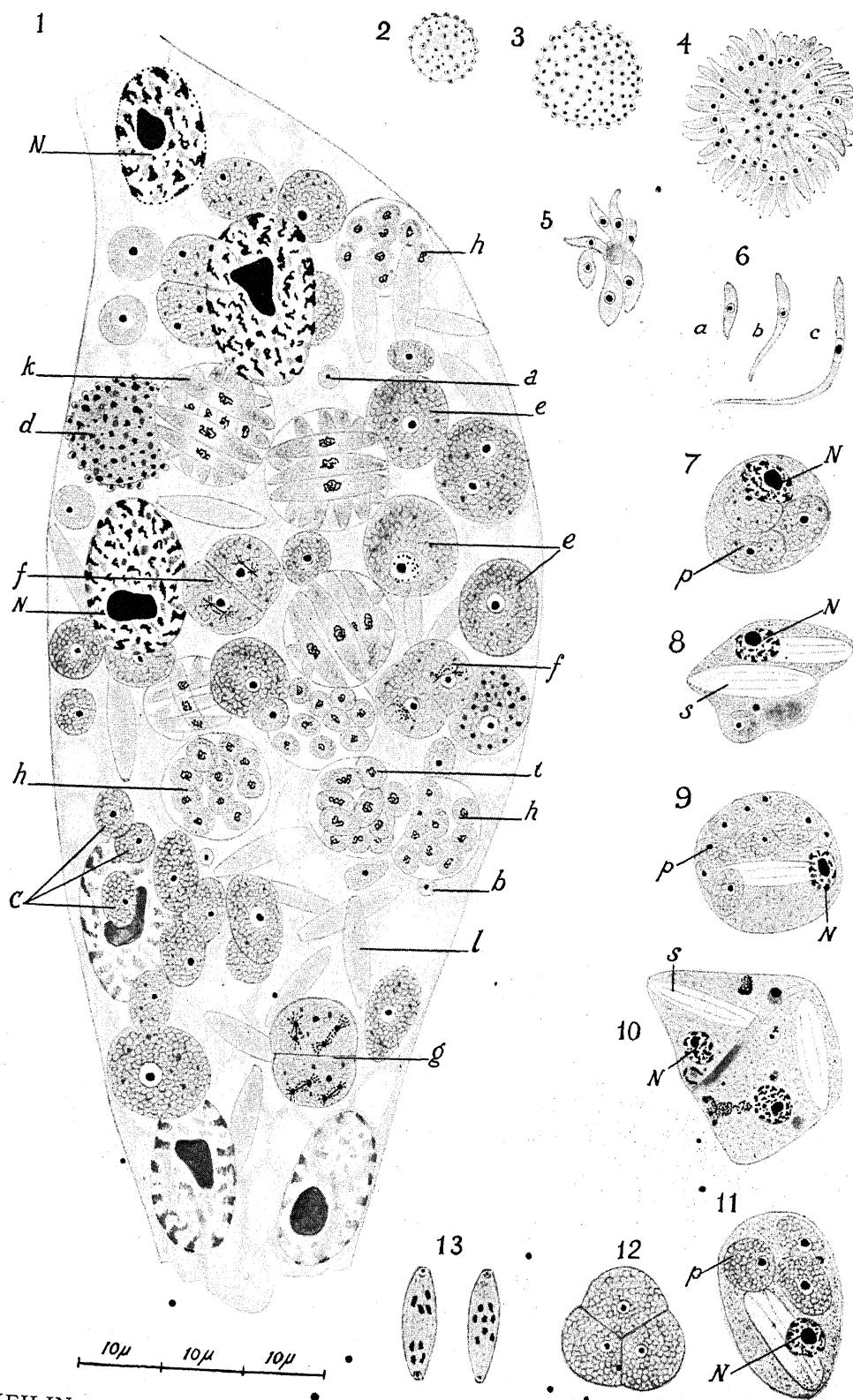
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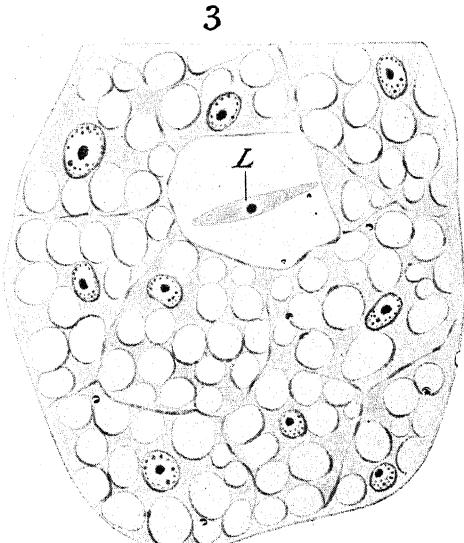
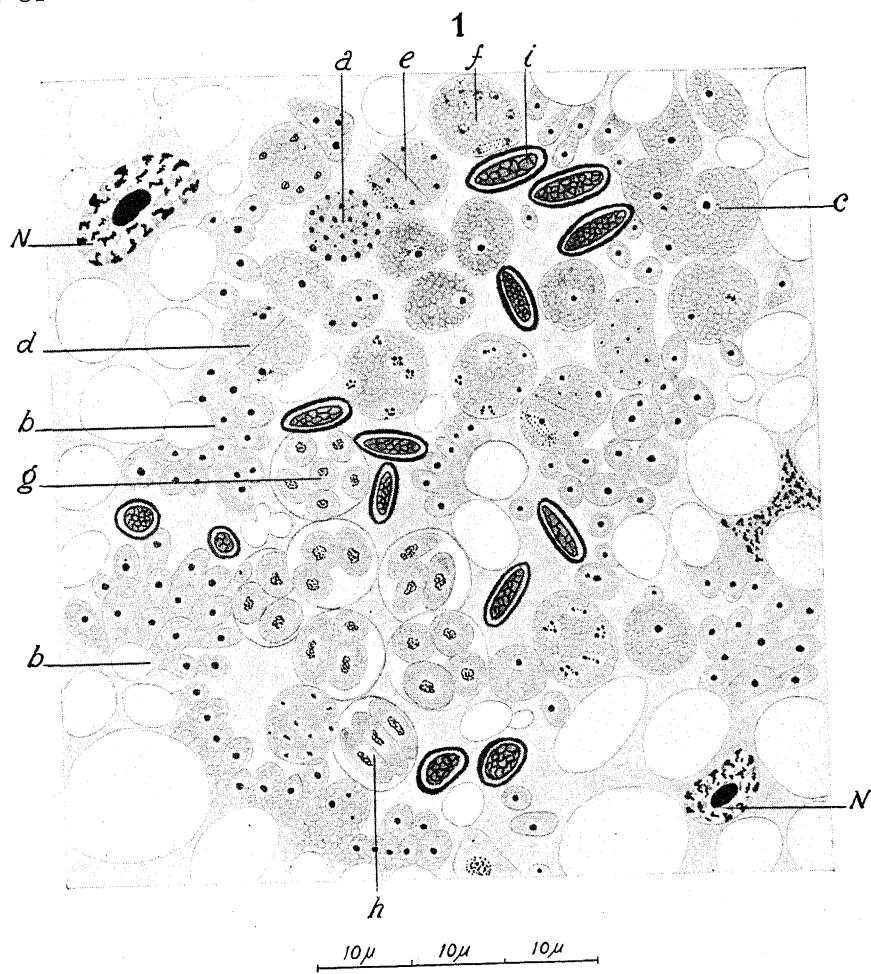
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VIII. EXPLANATION OF PLATES.

PLATE III.

Lipotropha macrospora.

All figures are of the magnification shown in scale at foot.

FIG. 1. A small portion of the fat body of *Systemus adpropinquans* (larva), fixed in Schaudinn and stained *in toto* in iron-haematoxylin, showing various stages of *Lipotropha macrospora*. This figure represents approximately one field of view, at high magnification ($\frac{1}{15}$ oil immers. + 6 compens. oc.). The parasites lying only near one horizontal plane have been represented: a. and b. young trophozoites, which develop into schizonts or gametocytes; c. more advanced stage of trophozoites; d. schizont slightly damaged in mounting; e. gametocytes; f. copula showing the beginning of the first spindle in each gametocyte; g. more advanced stage in formation of gametes; h. cysts with spherical sporoblasts; i. cysts with sporoblasts in a more advanced stage; k. cysts with sporocysts; l. sporocysts free in the fat body after breaking up of the cysts; N. nuclei of the fat cells.

FIG. 2. Young schizont, showing formation of merozoites.

FIG. 3. More advanced stage of schizogony.

FIG. 4. Ripe schizont with completely formed merozoites.

FIG. 5. Small group of merozoites after breaking up of schizont.

FIG. 6. Three stages of development of an active, elongated, gregarine-like stage of the parasite, from merozoites which are set free in the blood plasma of the host.

FIGS. 7-11. Leucocytes of the host with various stages of *Lipotropha*, which they have taken up after complete disintegration of the fat body. N. nucleus of leucocytes; s. spore of the parasite; p. various other stages of the parasite.

FIG. 12. Abnormal copula composed of three gametocytes.

FIG. 13. Sporocysts in longitudinal section, showing eight nuclei of sporozoites and two terminal basophile granules, one near each pole.

PLATE IV.

All figures are of the magnification shown in scale.

FIG. 1. Section of the fat body of *Systemus adpropinquans* (larva), showing various stages of *Lipotropha microspora*: a. schizont, showing numerous nuclei scattered throughout its protoplasm; b. young trophozoites surrounding a fat droplet; c. gametocytes; d. copula, showing the beginning of the first mitosis; e. and f. more advanced stages in formation of gametes; g. cyst with sporoblasts; h. cyst with sporocysts; i. sporocysts free in the fat cells; N. nuclei in the fat cells. The clear circular spaces represent fat droplets dissolved during preparation.

FIG. 2. A portion of the fat body of *Rhipidia ctenophora* (larva) *in toto*, showing L. elongated trophozoite stages of *Lipotropha microspora*.

FIG. 3. A small portion of the fat body of *Dasyhelea obscura* (larva), showing L. a parasite, probably *Lipotropha microspora*. The large clear space surrounding the parasite represents the dissolved fat.

THE DETERMINATION OF THE SALT ERROR OF INDICATORS AND THE ACCURATE ESTI- MATION OF THE *pH* OF SOLUTIONS BY COLORIMETRIC METHODS

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(With Four Text-figures.)

No attempt will be made in this paper to deal with the importance of the hydrogen ion concentration and of its regulation in vital processes. It is concerned solely with the colorimetric measurement of the *pH* by means of those indicators which change from one colour to another with variations in the *pH*. The use of indicators which either lose or gain in depth of colour with variations in the *pH*, such as phenolphthalein and the nitrophenols prepared by Michaelis and his co-workers, will not be referred to here.

Accurate measurement of the *pH* by colorimetric means may be said to date from the work of Sörensen. Sörensen showed that if certain substances be dissolved in water and these substances mixed in certain proportions then we obtain a mixture, the *pH* of which, if measured electrometrically, is found not to vary, provided the substances and the water in which they are dissolved is as pure as possible and the solutions are made of the same strength and mixed in the same proportions. The substances used by Sörensen in these solutions were substances which, when dissolved in water, possessed the property of requiring the addition of relatively large quantities of acid or alkali in order to change appreciably the *pH* of the solutions. Many substances are known to behave in this way and their action was compared by Sörensen to a "tampon," a word which has been translated into German as "puffer" and into English as "buffer." The solutions themselves are known as buffer solutions and the mixtures of the solutions as buffer mixtures. For the preparation of the solutions and the purification of the substances dissolved the reader is referred to Sörensen's original papers. The method of the colorimetric estimation of the *pH* is now very well known. Stock solutions, "buffer" solutions, are prepared, these are mixed in certain proportions and the *pH* of the mixtures is ascertained by reference to tables. These tables are, of course, prepared by determining, by measurement with the hydrogen electrode, the *pH* of the mixtures of the buffer solutions; but this need be done once only if we take the requisite care in the preparation of subsequent buffer solutions and mixtures. Then, to a certain quantity of a buffer mixture we add a certain quantity of indicator and, to a similar quantity of the solution whose *pH* we wish to determine, we add a similar

amount of indicator. If the tint of the two solutions is not exactly similar we then proceed to mix our buffer solutions in different proportions until, by trial, one is found which matches exactly the tint of the solution of unknown pH . Although the two solutions, the one having a known pH and the other an unknown pH , match in tint when similar quantities of the two liquids have been added to them the same amount of an indicator, it is not correct to say that the pH in the two liquids is the same. Certain corrections have to be made, and it is with these corrections that this paper chiefly deals.

Since Sörensen, many new buffer solutions and mixtures have been proposed, by Walpole, by Palitzsch, and by Clark and Lubs among others. If we assume that the chemicals from which these solutions have been made are pure and that the right quantities have been dissolved, then the attainment of any required pH is dependent on the accuracy of the proportions in which the solutions are mixed. It is usual to measure the quantities of each buffer solution from a graduated burette or pipette, and, even if we do not take any extraordinary precautions, accuracy to within 0.1 c.c. is easily maintained. If we take, for example, the borax-boric-acid mixtures of Palitzsch and plot the ratios of the borax solution as ordinates and the pH given by such mixtures as abscissae, we can then draw a curve which will tell us the pH of any given ratio or mixture. From the curve, to take an actual example, it is seen that the amounts required for mixtures differing from each other by 0.02 pH are easily measurable, even if we only make up so little as 20 c.c. of the buffer mixture. The actual amounts required of the two solutions to give a series of buffer mixtures between pH 7.70 and 7.60 differing from each other by no more than pH 0.02 are shown in the table below.

Table I

c.c. of Borax	c.c. of Boric	pH
3.0	17.0	7.60
3.1	16.9	7.62
3.2	16.8	7.64
3.3	16.7	7.66
3.4	16.6	7.68
3.5	16.5	7.70

It is quite easy, therefore, to prepare buffer mixtures differing from each other by pH 0.02. The question now arises as to how far these mixtures can be distinguished from each other after the indicator has been added, or, in other words, is the colour change of the indicator sensitive to these small differences of pH ? The question is easily answered. A dozen tubes each containing 10 c.c. of the buffer mixture and a similar quantity of indicator in each are prepared and sealed. The difference in pH between each tube is 0.02. The tubes are now marked by signs unknown to the observer and he is asked to arrange the tubes in their correct order. Under certain conditions and with certain indicators it will be found possible to arrange the tubes in their correct order, *i.e.* to read differences of pH 0.02 by means of indicators. These conditions may be stated as follows:

(1) The internal diameter of the tubes used for comparison of the mixtures with each other and with the solutions whose pH is unknown must be of nearly the same measurement. Extreme accuracy is not required as we are not concerned with depth of colour but with changes of tint produced by the mixture of two colours. A variation of 0.5 mm. either way in a tube of 15 mm. diameter will make no difference and test-tubes can easily be selected which vary in internal diameter much less than this.

(2) The transmitted light, by which the colour in the tubes is viewed, must of course be derived from the same source. Daylight from a north window is by far the best. The light from the window is allowed to fall on a piece of white paper on the table and the colour of the tubes, held at an angle of sixty degrees to the paper, is seen by transmitted light reflected from the paper.

(3) The tubes must be held side by side and not separated from each other as in a test-tube rack or in Walpole's colorimeter. This separation of the tubes from each other by a band of another colour is a hindrance to accurate matching. It is, however, of considerable assistance in matching the colours of the tubes if lines at a distance of 0.5 cm. apart be ruled on the paper from which the light is reflected. These lines seem to help to concentrate the eyes on two small and nearly adjacent portions of the tubes.

(4) For some reason or other the diluted solution of the indicator, as prepared from time to time from the same stock solution, may vary slightly and give apparently different tints which are almost impossible to match. It is necessary, therefore, to use for comparison only tubes containing indicator added from the same bottle of dilute solution.

(5) Different observers vary in their sensitivity to the changes of tint of the indicator. The colour change from yellow to red may be more easily detected than the change from yellow to blue and vice versa. But in any case it will be found that the eyes can be accustomed with training to detect much smaller differences of tint than is possible at first. Practice is as necessary here as in other things.

It is only in a certain number of indicators that a change of tint with a difference of 0.02 pH can be detected even if we observe carefully the conditions outlined above and train our eyes carefully. This property of indicators is called by Sörensen the virage. An indicator with a good virage is one that shows well-marked differences of tint or colour for small changes of pH . Generally speaking the indicators selected by Sörensen do not show such small differences of pH , for their virage is not sufficiently well marked. The indicators which we must use for this purpose are those selected by Clark and Lubs. These indicators are easily procurable nowadays from most drug-houses and manufacturing chemists. They are stable and the alkali salts of the indicators are easily soluble in water, nor do they precipitate from buffer solutions and mixtures. But their great advantage from the point of view of the accurate determination of the pH by colorimetric means is their excellent virage.

The range of an indicator is the extent of pH within the limits of which we can detect, by colour changes in the indicator, changes in pH . Thus the range of Cresol Red, as given by Clark and Lubs, is from pH 7.2 to 8.8. But at either end of this range

the indicator loses its virage and is not so sensitive to small differences of pH ; the virage is not, in fact, equally good over the whole range. Clark and Lubs are concerned only with detecting changes of 0.20 pH , and when we wish to detect changes of 0.02 pH the range of an indicator is curtailed but not to an extent which matters very much. The virage of Cresol Red is sufficiently good to detect changes of pH 0.02 between pH 7.65 and pH 8.45 . Above pH 8.45 Thymol Blue can be used between pH 8.4 and pH 9.2 . The indicators and the ranges within which I have been able to detect changes of 0.02 pH in the buffer mixtures are as follows:

Brom-Cresol-Purple.	Range from	pH 5.80 - 6.40
Brom-Thymol-Blue.	"	6.40 - 7.20
Phenol Red.	"	7.10 - 7.90
Cresol Red.	"	7.65 - 8.45
Thymol Blue.	"	8.40 - 9.20

The ease with which differences in tint can be distinguished varies with the different indicators. It is most difficult, I find, with Brom-Thymol-Blue, but it is just possible with practice. The virage, too, varies with the position of the pH in the range of the indicator, thus with Cresol Red it is possible to distinguish differences of pH 0.01 over a limited range of 7.80 to 8.05 .

With practice, then, and the observance of certain conditions it is possible to detect changes of pH of 0.02 in the buffer mixtures. It is now necessary to decide what corrections, if any, have to be applied after a solution of unknown pH has been matched exactly against a buffer mixture of known pH . It has long been known that salts and proteins in solution have a very considerable influence on the colour shown by an indicator. Thus if we match exactly by means of an indicator two solutions, the one containing half as much dissolved $NaCl$ as the other, we should, if we relied on the indicator alone, say that the pH in both was the same or, at any rate, within pH 0.02 . But if we measure the pH of these same two solutions by the electrometric method we find the pH to be different and the difference is far greater than pH 0.02 . The effect of the salt is to cause a depression of the pH , *i.e.* the solution with the higher concentration of salt is the more acid according to the electrometric measurement. The indicator does not cause a corresponding depression in the pH . It is assumed (although actual proof is lacking) that the more direct measurement of the hydrogen ion concentration by the electrometric method gives the correct result. It is therefore necessary to apply a correction to results obtained with indicators when the salt content of the solution, whose pH is desired, does not correspond exactly with that of the buffer mixture used for comparison. The correction which must be applied in order to bring the colorimetric determination into agreement with the electrometric one is called the salt error of the indicator. The presence of protein in solution also causes errors, but these errors are very difficult to correct. With the salt error it is otherwise. Recent work has provided us with a means of accurately estimating this error so that our colorimetric estimations of the pH can be made to correspond with the electrometric within 0.02 pH .

Sörensen was the first to realise the importance of the salt and protein errors. These errors vary with different indicators and they may be very considerable. Sörensen, in selecting his indicators, chose those which, in addition to possessing a good virage, did not possess very great salt or protein errors. The indicator which possesses the least salt error is Neutral Red¹, which is affected only by very great differences of salt concentration in the solutions compared colorimetrically. In ordinary work where great accuracy is not required the salt error of Neutral Red, which is no more than $pH 0.10$, is often neglected. The errors of all other indicators are however sufficiently large to make some correction necessary even in ordinary work. Sörensen's method of determining the extent of the salt error is the one which is generally used. A very dilute buffer mixture is prepared and divided into two portions to one of which is added a known quantity of NaCl or any other salt the effect of which on the indicator is to be determined. The pH of the two solutions is now measured both electrometrically and also by means of indicators. The concentration of the dissolved salts forming the buffer mixture is usually so small as to be negligible, and the mixture is assumed to be infinitely diluted. The corrections for the indicators shown in the table below have been established by Sörensen and Palitzsch, using this method.

Table II

Indicator	Buffer	Parts per 1000 of NaCl and corresponding errors			
		35	20	5	1
Para-nitro-phenol	Phosphate	+0.12	+0.08	—	—
Neutral Red	Phosphate	-0.10	-0.05	0.00	0.00
Alpha-naphtholphthalein	Borate	+0.22	+0.17	+0.03	-0.07
	Phosphate	+0.16	+0.11	-0.04	-0.13
Phenolphthalein	Borate	+0.21	+0.16	+0.05	-0.03

This method is undoubtedly an excellent one, but it suffers from the defect that it is exceedingly difficult to prepare and maintain constant buffer mixtures with a very low concentration of dissolved salts. The buffer mixtures so prepared are easily affected not only by the carbon dioxide of the atmosphere but also by substances dissolved from the glass of the container. In the case of Sörensen's and Palitzsch's determinations of the salt error it should be noted that, although the salt error is given to $pH 0.01$, nevertheless the virage of the indicators used does not permit a distinction being drawn of much less than $pH 0.03$.

The effect of the addition of various salts to the buffer mixtures and the dilution of the buffer mixtures themselves has recently been studied by Michaelis and Krüger. From their work it is quite clear that the depression of the pH by salts is caused by the metallic cation and not by the anion. Thus Michaelis and Krüger have shown that the addition of equivalent quantities of K_2SO_4 , KCl , and KBr to portions of the same buffer mixture produces a similar depression of the pH , but

¹ Kolthoff says Brilliant Yellow has no salt error at all, but the virage is too poor to make it a useful indicator.

the depression of the pH is not the same if equivalent quantities of KCl , $NaCl$, $LiCl$, or $RbCl$ are added to portions of the same buffer mixture. The figure given below, which is copied from Michaelis and Krüger's paper, shows the effect of adding different quantities of different salts to a buffer mixture which was prepared by considerably diluting the phosphate buffer mixture prepared according to Sörensen's directions by mixing equal quantities of an $M/15$ solution of primary potassium phosphate and an $M/15$ solution of secondary sodium phosphate.

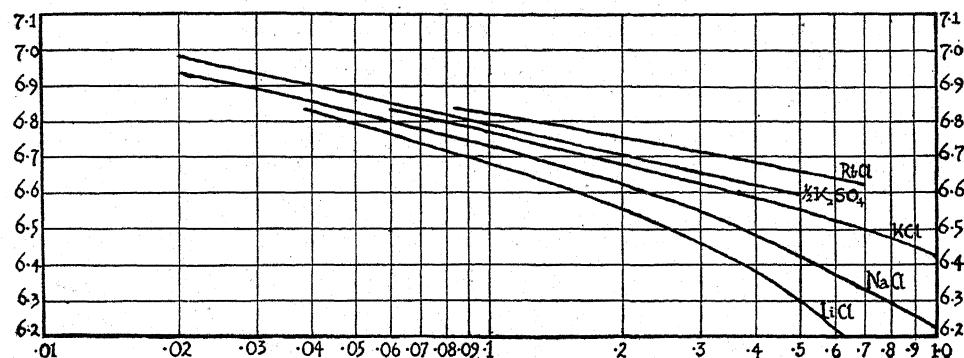


Fig. 1. Ordinates, pH . Abscissae, equivalent salt concentrations plotted on a logarithmic scale.

The dilution of a buffer mixture produces the opposite effect to the addition of salt, for the pH is raised by dilution. The curve (Fig. 2), plotted from Michaelis and Krüger's results, shows the effect of dilution on a buffer mixture of phosphates prepared as above from Sörensen's directions. The pH of the resulting mixture, when measured electrometrically, is 6.813. The pH of the mixture measured electrometrically at various dilutions are plotted as ordinates and the logarithms of the equivalent concentration of metallic kations are plotted as abscissae. In curve *A* the total equivalent concentration of the kations K and Na are plotted as abscissae, whereas in curve *B* the equivalent concentration of the Na ions only are plotted as abscissae. The points marked \times show the pH as found by electrometric measurement when $NaCl$ is added to a buffer mixture prepared as above and then diluted fifty times. The curve drawn through these points coincides with and prolongs curve *B*.

From the work of Michaelis and Krüger we can easily discover the salt error of our indicators which range from pH 6.60 to 7.00. The indicator selected by Clark and Lubs which falls within this range is Brom-Thymol-Blue. Solutions of the two phosphates prepared according to Sörensen's directions and mixed in equal proportions gave pH 6.81 when tested electrometrically. The same buffer mixture, diluted $1/25$, gave pH 6.87 when measured colorimetrically and, when $NaCl$ was added to the diluted buffer mixture to bring the concentration of Na to 0.6 Normal, the pH as measured colorimetrically was 6.56. From the curve (Fig. 2) we see that the electrometric measurements are, for $1/25$ dilution pH 7.07 and for a concentration of Na of 0.6 N pH 6.37. This gives a salt error, using the Sörensen phosphate

buffers for comparison and Brom-Thymol-Blue as the indicator, of -0.20 pH for a solution 0.0027 N for Na, and $+0.19$ pH for a solution 0.6 N for Na.

The work of Wells provides us with a means of determining the salt error of Cresol Red. Wells prepared a buffer mixture and added Cresol Red as an indicator. This buffer mixture coloured with Cresol Red was sealed up in a tube of resistance glass and used as a standard colour for comparison. Two solutions, one of Borax and one of Boric Acid, each containing 15 grams of the salt per litre, were prepared. From these solutions mixtures were prepared which, on the addition of indicator,

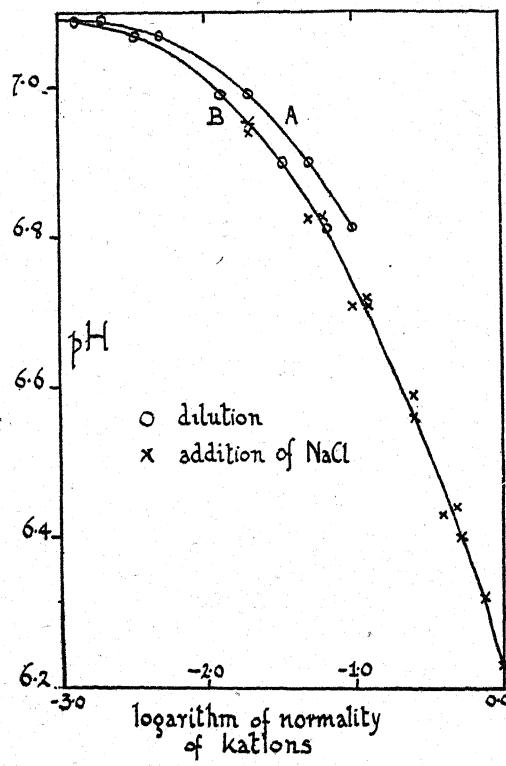


Fig. 2.

matched exactly the standard colour already mentioned. The pH of these mixtures, after matching the standard, was then determined electrometrically. The concentration of the salts in the mixtures was varied by dilution of the buffer solutions and also by adding NaCl to the mixtures. Wells was chiefly concerned with the preparation of a series of buffer mixtures in which the salt error for water containing 18 grams NaCl per litre was zero and of a table by which the error for different concentrations of NaCl could be allowed for. In this he is entirely successful. On the other hand, by an interpretation of the results which was not noticed by Wells himself, it is possible to draw a curve which shows the salt error of Cresol Red at any given concentration of salt and when compared with any buffer mixture of known composition and pH. Wells, when plotting his curves, took as ordinates pH

and, as abscissae, he plotted the logarithm to base 2 of the concentration of the dissolved salts in grams per litre of the mixture. The result is that the curve for dilution of the buffer mixture and the curve for the addition of NaCl form quite separate and distinct curves. If, however, we plot as abscissae the logarithms of the equivalent concentration of Na we obtain two curves which are continuous. This curve is reproduced as Fig. 3.

The colour of the indicator remains constant throughout. It is now obvious from the curve that, unless the solution whose pH is required and which is matched colorimetrically against the buffer mixture has exactly the same concentrations of Na ions, it will be necessary to apply a correction. If the concentration of Na ions in the solution whose pH is required is less than that of the buffer mixture with which

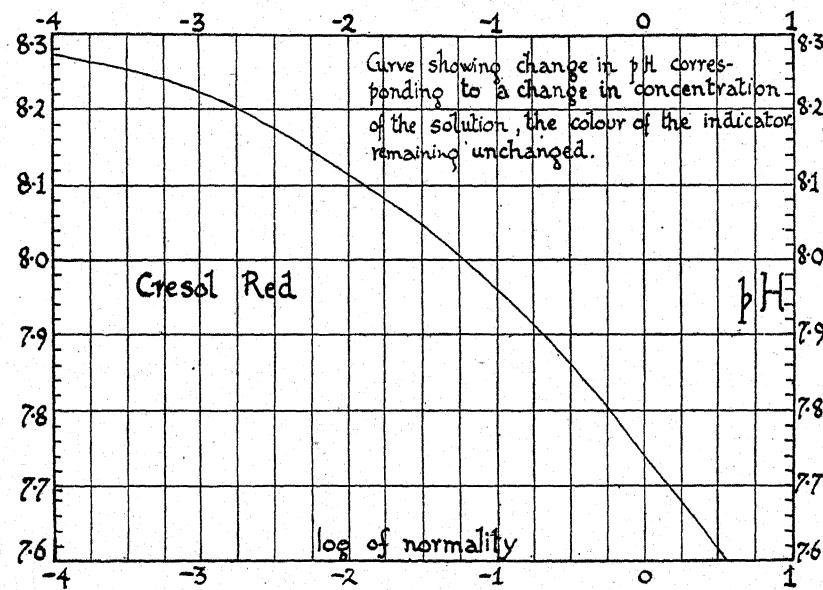


Fig. 3.

it is matched, then the pH of the buffer mixture is less than that of the solution matched against it. This correction, following Sörensen's notation, is denoted by a minus sign although it is added to the pH of the buffer mixture. Similarly, if the concentration of Na ions in the buffer is less than in the solution matched against it, the pH of the buffer mixture is too high. The correction is denoted by a plus sign and must be subtracted from the pH of the buffer. To take an example, supposing the concentration of the kations in our buffer mixture is $0.1 N$ and the solution whose pH is required is sea-water (normal sea-water is about $0.6 N$), then from the curve (Fig. 3) it is seen that a subtraction of 0.16 must be made from the value of the pH marked on the buffer mixture.

As will be seen it is important that the buffer solutions should, when mixed, always have the same equivalent concentration of metallic kations. Hitherto no attention has been paid to this and in none of the buffer mixtures commonly used,

except Walpole's acetic acid sodium acetate mixtures, is the equivalent concentration the same in all the mixtures. In some mixtures, such as Sörensen's phosphates and Palitzsch's borax-boric-acid mixtures, the molecular concentration of the dissolved salts remains the same in all mixtures. In the Clark and Lubs buffers, which are very convenient, no attempt has been made to preserve the same concentration, either molecular or equivalent, in all the mixtures, but the variations are so slight as to be immaterial except where very accurate work is required. But as, in this paper, accuracy is the chief desideratum it is necessary to consider the varying changes in a buffer mixture such as those recommended by Clark and Lubs. Clark and Lubs take 50 c.c. of a solution which is 2N for Potassium and, after adding amounts of 2N NaOH which vary from 0.40 c.c. to 47.00 c.c., the resultant mixture is diluted to 200 c.c. The buffer mixtures therefore vary in equivalent concentration of kations from 0.097N to 0.050N , so that a salt error, due to changes in the concentration of the buffer mixture, up to as much as 0.05pH , must be taken into account when it is desired to read the pH by colorimetric means accurate to 0.02pH . Allowance has also to be made for that Potassium ions at a concentration of 0.05N depress the pH by 0.02 more than Sodium ions at the same concentration.

From the curve, Fig. 3, it is seen that the salt error of Cresol Red using Clark and Lubs buffer mixtures will, for normal sea-water, vary from $\text{pH } 0.16$ to 0.21 according to the concentration of the buffer mixture, with a mean value of $\text{pH } 0.185$. Now McClendon has determined, both electrometrically and colorimetrically, the pH of sea-water saturated with carbon dioxide at a given tension. By following his directions, solutions of borax, boric acid and NaCl may be prepared which, on mixing and adding indicator, show the electrometric values of the pH of sea-water which matches them exactly in tint. In this way, by preparing McClendon's solutions and comparing them with Clark and Lubs mixtures, Atkins has found the salt error of Cresol Red with Clark and Lubs mixtures to be $+0.18$. I myself have prepared these same solutions and compared them with Clark and Lubs mixtures, the pH of which was checked electrometrically. As a result, I find that the salt error with a boric acid KCl and NaOH mixture (Clark and Lubs), the pH of which is 8.40 and the concentration of which is nearly 0.06N , is $+0.19$, while the error as given by the curve should be 0.20 . This is a striking corroboration of the value of the curve in accurately defining the salt error of Cresol Red when we know the equivalent concentration of kations in both the buffer mixture and the solution whose pH is required.

We have now shown that the salt error of Cresol Red can be accurately determined from the curve (Fig. 3), or, in other words, we can bring the pH , as determined by the aid of Cresol Red, into accurate agreement with the values found electrometrically. Starting from Cresol Red it can now be shown that it is possible to determine the salt errors of all other indicators with equal accuracy. Advantage is taken of the fact that the range of the indicators just overlaps. A solution of known equivalent salt concentration, but different from that of the standard buffer mixtures used for comparison and having a pH near that where the range of the

indicators overlaps, is prepared and divided into two portions. The pH of one portion is determined accurately by the indicator whose salt error is known, in this case Cresol Red, and the pH of the other portion is determined by the indicator whose range overlaps that of Cresol Red. In both cases the same series of buffer mixtures of known pH is used for comparison. If the salt errors are the same, measurement of the pH as given by the different indicators will be the same. If they differ, then the difference must either be added or subtracted from the known salt error of Cresol Red in order to give us the salt error of the other indicator.

The preparation of dilute buffer solutions of different *pH* to which salt can be added in varying quantities is a matter of little difficulty if we use for this purpose solutions of carbonates saturated with carbon dioxide at different tensions. Henderson has shown that a bicarbonate solution of known concentration and at a given temperature when saturated with carbon dioxide at a known tension has a definite *pH*. If, therefore, we prepare two solutions of sodium bicarbonate of known concentration and saturate them with carbon dioxide at a similar tension, the *pH* of both the solutions will be the same. Hasselbalch has made a series of accurate determinations of the *pH* of solutions of sodium bicarbonate in contact with carbon dioxide at a known tension, and as a result he finds that the *pH* is given by the formula

where B_{ik} = equivalent concentration of the NaHCO_3 ,

and pK_1 = a constant the value of which varies with the equivalent concentration of the NaHCO_3 .

The equivalent concentration is easily determined, either by weighing out a quantity of pure sodium carbonate and dissolving it in water which is afterwards saturated with carbon dioxide or by titrating the solution with acid of known strength, using Methyl Orange as an indicator.

The equivalent concentration of carbonic acid is calculated from the tension of carbon dioxide. Hasselbalch found that water saturated with carbon dioxide at a tension of 91.7 mm. of mercury reacted as an acid, the strength of which was 0.1 N . If, therefore, the tension of the carbon dioxide, measured in millimetres of mercury, be multiplied by $\frac{0.1}{91.7}$ the result gives the equivalent concentration of carbonic acid. The tension of the carbon dioxide is measured by analysing samples of the carbon dioxide mixture in a Haldane apparatus. The values of pK_1 for different concentrations of sodium bicarbonate at a temperature of 18°C are given by Hasselbalch as follows:

Conc. of bicarbonate	pK_1
0.05	6.43
0.01	6.53

The method of procedure is now as follows: supposing we wish to produce a

buffer solution of bicarbonates in contact with carbon dioxide, the pH of which shall be about 7.70. A solution of sodium bicarbonate, about 0.005 N , is prepared. Into four clean test-tubes of the same diameter as those tubes used for colorimetric comparisons, 5 c.c. of the bicarbonate solution are placed. By means of the apparatus shown in Fig. 4 air containing carbon dioxide is bubbled through the solutions in the tubes. Cresol Red is added to tubes 1 and 4. The air bubbling through the tubes contains a constant proportion of carbon dioxide and therefore the tension of carbon dioxide in the tubes is, if the amount of carbon dioxide be small, the same in all. Saturation of such a small quantity as 5 c.c. is very easily attained, and after bubbling the air through the tubes for from ten to twenty minutes it will be seen that the colour in tubes 1 and 4 is the same and, moreover, that further bubbling does not change the colour. Saturation is therefore complete. The carboy is filled in the first instance with fresh air from outside the building. The pH given by saturation of the bicarbonate solution with this air, which contains about 3.0 parts per 10,000 of carbon dioxide, may be too high. In this case the bung of the carboy is removed and the proportion of carbon dioxide in the carboy is increased by breathing into it. Complete mixture of the carbon dioxide with the air is ensured by shaking the carboy. This mixture is then bubbled through the solutions in the tubes. With a little practice it is quite easy to adjust the proportions of carbon dioxide in this simple manner so that the pH of the solutions is very nearly that required. Having thus prepared two buffer solutions of exactly similar pH , they are now measured colorimetrically both by Cresol Red and Phenol Red. To the bicarbonate solutions NaCl may be added in different quantities and the pH measured in the same way. As a result we find that Cresol Red and Phenol Red have the same salt error at concentrations of Na below 0.1 N , but above this the salt error of Phenol Red is slightly less than that of Cresol Red. Kolthoff has determined the salt error of Phenol Red and finds it to be $+0.15$ with a concentration of $\text{NaCl } 0.5\text{ N}$ and using Sörensen's phosphate buffer mixtures for comparison, while by my method I find the salt error under the same conditions to be 0.16 . In the same way the salt errors of all the other indicators can be measured. I have found that the salt error of all the sulphonphthalein indicators investigated, viz. Thymol Blue, Phenol Red, Brom-Thymol-Blue and Brom-Cresol-Purple, have the same salt error as Cresol Red at concentrations of NaCl below 0.1 N . I have also determined the salt error of Neutral Red and find that, using Clark and Lubs buffer mixtures, there is no salt error at all lesser concentrations of salt than the buffer mixture, *i.e.* below 0.1 N . It is therefore a very valuable indicator for fresh-water. For concentrations of salt above that of the buffer mixture there is a slight error. For sea-water, 0.6 N for NaCl , I find that the salt error is 0.12 using Clark and Lubs buffer mixtures. At concentrations above 0.1 N the salt errors of Brom-Thymol-Blue and Thymol Blue do not differ from those of Cresol Red by more than 0.01 pH . For Phenol Red the salt error, at a concentration of $\text{NaCl } 0.6\text{ N}$, is 0.03 less than that of Cresol Red, while for Brom-Cresol-Purple it is 0.07 more. If the concentration of the buffer mixture is 0.08 N , which is the average value of most of the common buffer mixtures, then the salt errors of the four sulphonphthalein indicators are as follows:

Table III

Indicator	Conc. of buffer mixture	Conc. of solution compared	Salt error
Brom-Cresol-Purple	0.08 N	0.6 N	+0.25
Brom-Thymol-Blue	0.08 N	0.6 N	+0.19
Phenol Red	0.08 N	0.6 N	+0.15
Cresol Red	0.08 N	0.6 N	+0.18
Thymol Blue	0.08 N	0.6 N	+0.18

A striking example of the accuracy of colorimetric determinations of the pH by the sulphonphthalein indicators is provided by determinations of the pH of solutions of bicarbonates of known strength, saturated with carbon dioxide at a known tension

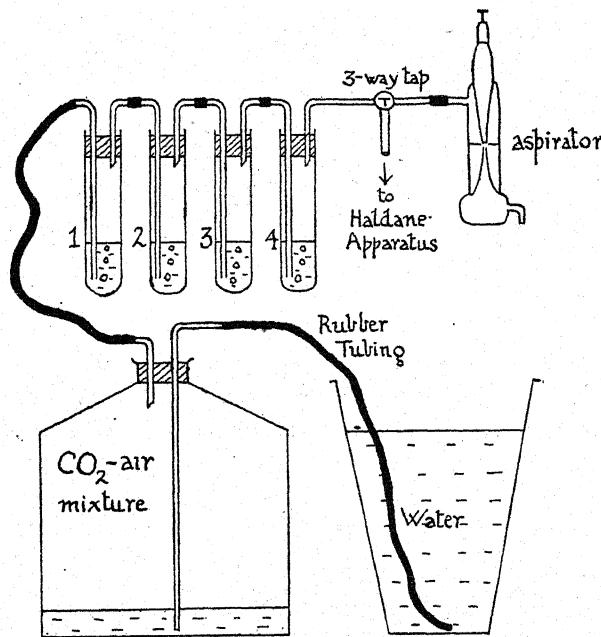


Fig. 4.

and at a given temperature. The apparatus I used was that shown in Fig. 4. A sample of the air, after bubbling through the solution, was withdrawn and analysed in a Haldane apparatus to find the proportion of carbon dioxide. If now we know the pH , the tension of carbon dioxide, and the strength of the bicarbonate solution and the experiment has been performed at a uniform temperature of 18° Centigrade, then we can from equation (1) above find the value of pK_1 . The value of pK_1 has been found by Hasselbalch by careful measurements with the hydrogen electrode and, accepting these as correct, we can test our colorimetric determinations against these. The results of such a test are given below in Table IV. The buffer mixtures used for comparison were the borax-boric-acid mixtures of Palitzsch, the salt error of which, for a solution $0.01 N$ for Na, is given by the curve (Fig. 3) as -0.12 .

Table IV

Conc. of NaHCO ₃ solution	Tension of CO ₂ in mm. of Hg	Temp. °C.	pH corrected for salt error	pK ₁
0.01 N	5.15	18	7.78	6.54
0.01 N	2.54	18	8.10	6.54
0.01 N	2.95	18	8.04	6.53

The value of pK_1 as determined by Hasselbalch for 0.01 N sodium bicarbonate is 6.53 at a temperature of 18° Centigrade. It is therefore possible, providing our buffer mixtures used for comparison are correct and using the curve given in Fig. 3 for determining the salt error, to estimate the pH of a solution to within 0.02 of the electrometric measurement of the same.

Further, it is seen from the curve (Fig. 3) that, since the salt error of Brom-Thymol-Blue is the same as that of Cresol Red, the salt error, using a Sörensen phosphate buffer mixture N/15 for Na, will be -0.18 for a solution 0.0027 N and +0.21 for a solution 0.6 N. The results obtained by the colorimetric measurement of the pH of diluted buffer mixtures without and with the addition of NaCl (see pp. 35 and 36) give the salt errors as -0.20 and +0.19 respectively. The agreement is a close one and provides a convenient check on the results.

SUMMARY.

1. It is shown that, using the sulphonphthalein indicators recommended by Clark and Lubs, it is possible to detect differences in pH of no more than 0.02.
2. A curve is deduced by means of which it is possible to determine the salt error of Cresol Red for any difference of concentration between the buffer mixture and the solution of unknown pH.
3. A simple method of determining the salt error of any other indicator by comparison with Cresol Red is outlined.

I am indebted to Dr C. G. L. Wolf for checking by means of a hydrogen electrode the pH of my buffer mixtures.

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THE MEASUREMENT OF THE CARBON DIOXIDE OUTPUT OF FRESH WATER ANIMALS BY MEANS OF INDICATORS

By J. T. SAUNDERS, M.A.

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Demonstrator of Animal Morphology.

(With One Text-figure.)

FROM theoretical considerations Prideaux has shown that the total CO_2 content of a solution of carbonates or bicarbonates or a mixture of both has a very definite relation to the hydrogen ion concentration. For dilute solutions this relationship is given by the formula

$$R = \frac{1 + 2 \frac{k_2}{[\text{H}']} + \frac{k_w \{k_1 + [\text{H}']\}}{[\text{H}'] k_1 C}}{1 + \frac{k_2}{[\text{H}']} + \frac{[\text{H}']}{k_1}},$$

where $k_1 = 3.04 \times 10^{-7}$, the dissociation constant of the first stage of dissociation of carbonic acid,

$k_2 = 6.0 \times 10^{-11}$, the dissociation constant of the second stage of dissociation of carbonic acid,

$k_w = 0.7 \times 10^{-14}$, the dissociation constant of water,

$[\text{H}']$ = the hydrogen ion concentration of the solution,

C = the total concentration of alkali, expressed as equivalents per litre,

and $R = \frac{\text{equivalent of alkali}}{\text{molecules of carbonic acid}} \text{ or } \frac{\text{equivalent concentration of alkali}}{\text{molecular concentration of } \text{H}_2\text{CO}_3}$.

In the above equation k_1 , k_2 and k_w are constant, $[\text{H}']$ and C are easily measured and from these we obtain the value of R . But, as the solving of this equation is rather laborious, I have therefore tabulated the values of R which correspond to different values of $-\log \text{H}'$ (or pH) at equivalent concentrations of $0.01 N$, $0.001 N$ and $0.0001 N$. From the values of R thus found we can find the total CO_2 , measured at N.T.P., which corresponds with the given pH at the given equivalent concentration. The total CO_2 at the same pH in a solution of different concentration from those shown in the table is found by multiplying the total CO_2 for this pH , as given by the table, by the ratio of the equivalent concentrations. Thus if the total CO_2 , in c.c. at N.T.P. per litre, for a solution $0.001 N$, is 22.85 at $\text{pH } 8.00$, then, at the same pH in a solution 0.0025 the total CO_2 will be

$$22.85 \times \frac{0.0025}{0.001} = 57.125.$$

Table I

Table showing the value of R and the amount of CO_2 in c.c. at N.T.P. per litre at a given pH and at equivalent concentrations of alkali $\cdot 01 \text{N}$, $\cdot 001 \text{N}$ and $\cdot 0001 \text{N}$.

pH	R			Total CO_2 in c.c. at N.T.P. per litre		
	$\cdot 01 \text{N}$	$\cdot 001 \text{N}$	$\cdot 0001 \text{N}$	$\cdot 01 \text{N}$	$\cdot 001 \text{N}$	$\cdot 0001 \text{N}$
9.00	1.053	1.065	1.119	211.0	20.85	1.985
8.70	1.022	1.025	1.055	218.2	21.70	2.11
8.50	1.005	1.007	1.027	221.5	22.10	2.18
8.25	·993		1.005	224.0	22.40	2.215
8.00	·974		·982	228.5	22.85	2.27
7.70	·941		·945	237.0	23.70	2.36
7.50	·907		·909	245.5	24.55	2.46
7.25		·845		263.0	26.30	2.63
7.00		·752		296.0	29.60	2.96
6.75		·600		371.0	37.10	3.71
6.50		·494		451.0	45.10	4.51
6.30		·395		564.0	56.40	5.64
6.00		·232		961.0	96.10	9.61

If a curve be plotted from the values given in this table the total CO_2 at any given pH can then be obtained for three solutions having equivalent concentrations of alkali of $\cdot 01 \text{N}$, $\cdot 001 \text{N}$ and $\cdot 0001 \text{N}$. The slope of all three curves will be similar from $\text{pH } 6.0$ to $\text{pH } 7.5$ and the slope of the curves for $\cdot 01 \text{N}$ and $\cdot 001 \text{N}$ will not differ until $\text{pH } 8.45$ is reached. All three curves are therefore best drawn on a single sheet of squared paper.

For the experimental proof of the correctness of these equations Prideaux relies on two observations of Walker and Kay. Walker and Kay observed the pH of a dilute solution of calcium carbonate when saturated with normal fresh air (i.e. at a tension of $\text{CO}_2 = \frac{100^3}{100000} \times 760 \text{ mm. Hg.}$) using Azolithmin as an indicator and Sörensen's phosphate buffers for comparison. No account is taken by Walker and Kay of any salt error in the indicator and it should also be noted that the excess of carbonic acid that is in equilibrium with the pressure of CO_2 in the atmosphere has to be calculated from the equation of homogeneous equilibrium, it was not found experimentally. Fortunately, much more convincing proof is now available of the correctness of these equations for dilute solutions. I have determined accurately the pH of a solution of tap-water diluted with distilled water to $\cdot 0025 \text{N}$. Mr Barcroft has then extracted all the CO_2 from a known amount of the sample and measured the volume thus extracted. The pH of the solutions was measured colorimetrically by the method which I have described in this Journal, p. 3c.

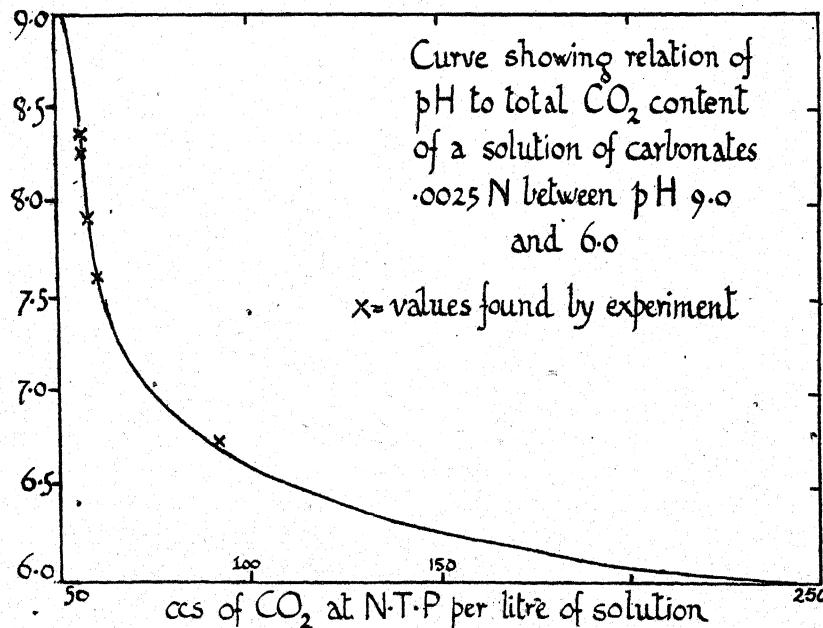
The CO_2 present in the sample was measured by sucking up about 100 c.c. into an exhausted vessel. Excess of recently boiled dilute H_2SO_4 , more than sufficient to neutralise the carbonates in the water, followed the sample into the exhausted vessel. The exhausted vessel was surrounded with a jacket of warm water and the contents boiled. The CO_2 evolved on the addition of acid and by boiling was

pumped off with a vacuum pump and all the gas so evolved, together with the oxygen and nitrogen from the dissolved air in the sample, was collected in a burette over mercury. From this burette it was transferred to a Haldane apparatus and the CO_2 measured by absorbing it with KOH. Afterwards the volume of CO_2 found was reduced to c.c. at N.T.P. per litre of solution. The values thus found experimentally are given below together with the values calculated from Prideaux's equations.

Table II

Concentration of carbonate expressed as equivalents of CaCO_3 per litre of sample	pH	Tempera- ture of sample °C.	Amount of sample taken for analysis in c.c.	Total CO_2 abstracted from sample analysed at temp. stated	c.c. at N.T.P. of CO_2 evolved per litre of sample	
					Found	Calculated
.0025	8.36	14	99	5.80	55.7	55.7
.0025	8.26	14	97	5.70	56.0	55.9
.0025	7.91	13	93	5.62	57.5	57.5
.0025	7.61	13	86	5.48	60.2	60.2
.0025	6.72	14	73	6.89	91.3	90.0

As will be seen, from the table above, the correspondence between the calculated amounts of CO_2 and those actually found is very good, except at the lowest pH 6.72. The reason for this is given by the curve below, which may be calculated from the values given in Table I.



It is not possible to read the *pH* colorimetrically more accurately than ± 0.02 . Above *pH* 7.0 this error will not give rise to any serious errors in estimating the CO_2 content of the solution. Below *pH* 7.0 the error in the CO_2 content, estimated from the *pH* of the solution, will be ± 1.5 per cent. The best results in accurately estimating the CO_2 output of fresh water animals will thus be attained by working between the limits *pH* 7.5-8.5.

METHODS AND APPARATUS.

For the method of reading the *pH* of a solution to within 0.02 by the use of sulphonphthalein indicators and for the accurate estimation of the salt error reference must be made to my previous paper in this Journal. In order to estimate the CO_2 output of a fresh water animal advantage is taken of the fact that the sulphonphthalein indicators are not poisonous at the dilution at which they are used, nor are they absorbed by the tissues of the animal. If, therefore, an animal be placed in a dilute solution of carbonates together with some indicator the CO_2 evolved by the animal will be dissolved in the water. This solution of the CO_2 evolved will cause the water to become more acid, and this will be reflected as a colour change in the indicator. Provided the solution in which the animal is placed be kept gently agitated and at the same time protected from the air so as to prevent both loss or gain of CO_2 to or from this source, the *pH* as measured by the indicator will form a continuous record of the CO_2 output of the animal.

The relation between the size of the animal and the amount of water in which it is confined will decide whether the colour change of the indicator be a slow or a rapid one. But as, in practice, 2-3 c.c. is the minimum amount of water in which the colour changes can be observed, the colour change of the indicator will, in the case of very small animals, be very slow unless numbers are crowded together in the same water.

In order to protect the solutions from contact with the air thick paraffin oil (the kind prepared according to the British Pharmacopœia and used as an aperient) serves very well. At the same time this method of sealing allows the solution to be gently agitated without any fear of breaking the seal.

As a practical example of the measurement of the CO_2 output of a fresh water animal I will give the method of measuring the respiration of *Zoarces viviparus*, the "Millions" fish, which lives in tropical waters, and also of eggs of *Rana temporaria*, the common English frog. First a solution of Na_2CO_3 , 0.01 *N*, is prepared by weighing 0.53 gram of Na_2CO_3 and dissolving this in a litre of distilled water. 10 c.c. of this solution are then diluted to 100 c.c. with distilled water and the strength of the resultant solution checked by titrating with 0.1 *N* H_2SO_4 , using Methyl Orange as an indicator. Portions of this solution are now measured into test-tubes, such as are used for comparison of indicators, and every portion of solution is saturated with fresh air taken from outside the building. The method of doing this has been described in my previous paper in this Journal. The solution in the test-tubes thus saturated will, at a temperature of 18° C., have a *pH* of 8.20. The indicator, Cresol Red, is now added, then the animal is placed in the solution

Measurement of Carbon Dioxide Output in Fresh Water Animals 47

and finally one or two c.c. of oil are floated on top of the water. The temperature of the solutions is maintained constant by placing the test-tubes in a water bath. Observations of the *pH* are made from time to time and the results recorded.

The following gives the details of three typical experiments, two on *Zoarces viviparus* the other on a number of frog's eggs:

Expt. 1

Date: 1 Mar. 1923.

Animal: *Zoarces viviparus* (larva). Weight: 1.079 gr. Solution: 0.001 N Na_2CO_3 . Amount of solution used: 10 c.c. Temperature maintained during experiment: 26°C. Indicator: Phenol Red.

Time	...	11.17½ to 11.18½	11.18½ to 11.20½	11.20½ to 11.22½	11.22½ to 11.26	11.26 to 11.30½	11.30½ to 11.32½	11.32½ to 11.36	11.36 to 11.47	11.47 to 12.06
Change in <i>pH</i> corrected for salt error		8.29 to 8.14	8.14 to 7.94	7.94 to 7.74	7.74 to 7.54	7.54 to 7.34	7.34 to 7.24	7.24 to 7.14	7.14 to 7.04	7.04 to 6.94
CO_2 output per gram per minute		.0187	.0248	.0248	.0213	.0238	.0372	.0318	.0119	.0089

Expt. 2

Date: 4 Mar. 1923.

Animal: *Zoarces viviparus* (larva). Weight: 1.472 gr. Solution: 0.00013 N Na_2CO_3 . Amount of solution used: 10 c.c. Temperature maintained during experiment: 26°C. Indicator: Brom-Cresol-Purple.

Time	...	11.23½ to 11.27	11.27 to 11.28½	11.28½ to 11.33	11.33 to 11.45½	11.45½ to 12.22½
Change in <i>pH</i> corrected for salt error		7.42 to 6.67	6.67 to 6.44	6.44 to 6.27	6.27 to 6.10	6.10 to 6.0
CO_2 output per gram per minute		.0375	.068	.0184	.0152	.0037

Expt. 3

Date: 26-27 Mar. 1923.

Animal: Egg of *Rana temporaria* (gastrulation just completed). Weight: 0.0282 gr. Solution: Tap water diluted to 0.0025 N. Amount of solution used: 5 c.c. Temperature during experiment: 14°C. Indicator: Cresol Red.

Time	...	13.15 to 20.15	13.15 to 20.15	13.15 to 20.15	20.15 to 10.45	20.15 to 10.45	20.15 to 10.45
Change in <i>pH</i> corrected for salt error		8.50 to 8.30	8.50 to 8.30	8.50 to 8.30	8.30 to 8.05	8.30 to 8.05	8.30 to 8.00
CO_2 output per gram per minute		.0028	.0028	.0028	.0020	.0020	.0025

Except in Expt. 2 the readings are fairly consistent. In Expt. 2 the solution, when saturated with fresh air, gave, by reason of its extreme dilution, too low a

pH at the commencement of the experiment, and sufficiently accurate estimation of the CO_2 at short intervals was therefore impossible. On the other hand, the CO_2 output per minute per gram during the duration of the experiment is very nearly the same in Expt. 2 as in Expt. 1 (0.0164 c.c. CO_2 at N.T.P. per litre in Expt. 2 and 0.0174 c.c. CO_2 at N.T.P. per litre in Expt. 1). In both cases the CO_2 output falls off towards the end of the experiment, due doubtless to the decrease in the amount of oxygen available.

It is, therefore, most convenient to use for these experiments a solution about 0.0025 N for carbonates, which, by the simple expedient of bubbling fresh air through it, can be brought to pH 8.60 at 18°C . (a higher temperature increases and a lower temperature decreases the pH by approximately 0.01 for every degree centigrade). Then, as the pH falls, the CO_2 content can be accurately estimated at short intervals. In order to prove that CO_2 , and nothing else, has affected the pH during the experiment, the animal is removed after the experiment is complete and fresh air is again bubbled through the solution. The effect of this is to cause the pH to return to its original value. Fresh air provides us with a CO_2 mixture of nearly constant composition. The possible variation in the pressure of CO_2 in fresh air taken from the same source on different days will cause a variation in the pH of 0.05, other conditions remaining constant. The variations from hour to hour in the pressure of CO_2 in the atmosphere are so small as to be measurable only with difficulty and will have no effect on the pH .

I should like here to express my thanks to Mr J. Barcroft, F.R.S., for his kindness in extracting and measuring for me the CO_2 content of the solutions given in Table II.

SUMMARY.

Tables are given showing the relationship of the pH of a dilute solution of carbonates to the total CO_2 content. Animals are placed in such dilute solutions which are coloured with indicators, contact with the atmosphere is prevented by a layer of oil and the pH as measured by the changing tint of the indicator records also the output of CO_2 . An output of CO_2 of no more than 0.0001 c.c. is easily and accurately measured.

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SPECIFIC GRAVITY AS A FACTOR IN THE VERTICAL DISTRIBUTION OF PLANKTON

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(With Two Text-figures.)

ALL theories advanced hitherto to account for the vertical distribution of species have attempted to explain these diurnal movements as dependent entirely on changes in the environment, to which the animal responds by certain definite "tropisms."

G. H. Parker quotes the work of Jacques Loeb who, studying copepods (*Temora longicornis*) at Woods Hole, found that they could be made negatively phototropic by increasing the temperature of the water, and positively phototropic by decreasing the temperature. He calls attention to positive and negative geotropism as a factor in determining vertical distribution. Parker stresses this point and affirms that change of temperature is the only effective means of changing the nature of the geotropic response. Parker worked on *Labidocera aestiva*, and offers an explanation of the depth migrations. He suggests that the female forms rise to the surface with the setting of the sun because they are positively phototropic to faint light and negatively geotropic, and they descend at sunrise because they are negatively heliotropic to strong light; the male forms follow the females in migration because they are positively chemotropic to the female.

C. Juday⁽²⁾ observed the movements of plankton Crustacea in great lakes, and discusses the diurnal movements in correlation with variations of food supply, temperature and light. He concludes that food supply cannot be a potent factor, as whereas the phytoplankton is found most abundantly near the surface, some forms are equally well supplied with food at greater depth; and also in Lake Mendota, *Daphne pulicaria* remained in the region of the thermocline day and night, where food was not so abundant as near the surface.

With regard to temperature, Juday affirms that his own observations do not support Ostwald's suggestion, that the descent of the plankton forms is the purely mechanical result of the lowering of viscosity of the water through increase of temperature, but that the upward movement is achieved by promiscuous wandering. Juday considers that change of light intensity is the most potent factor, although not the only one, and that in the main the depth migrations are the expression of the animal heliotropism.

If the animal and its environment are considered from a purely mechanical point of view, it becomes apparent that changes in the density of the animal itself must be of importance as well as changes in the density of the water, and that if

there is any rhythmic change in the specific gravity of the animal, this must be a factor in determining the vertical distribution of the species.

The equation given by Stokes for the fall of a sphere through water is

$$V = \frac{2r^2(s - s')}{9\eta} g$$

where V = velocity,

r = radius of the sphere,

s = density of sphere,

s' = density of water,

η = viscosity of the water.

For the moment considering the plankton animal as a sphere, it is seen that its velocity can be expressed in terms of its dimensions, its density, and the density and viscosity of the water.

If the animal is capable of expending a certain amount of energy which keeps it at a certain depth, and its specific gravity is increased, it would be expected that its distribution in depth would alter so as to keep constant the value of the right-hand side of the equation. That is to say, the change in density of the animal is a factor in determining the depth migrations. A number of experiments were done to attempt to detect some such rhythmic change in density.

MATERIAL AND METHODS.

The material used was *Daphne pulex* which was first found in abundance in the pools on Sheeps' Green, Cambridge, and later in the pond at Girton College, Cambridge.

The method of investigation consisted in narcotising the animals with a 1 per cent. solution of urethane, and observing the rate of fall down a long vertical tube of water. A scale was fixed to the tube and the tube immersed in a tank of running water at constant temperature. The time taken by the animal to fall 20 cms. after attaining a uniform velocity was ascertained by means of a stop-watch; from these data the velocity was calculated.

It was found that *D. pulex* narcotised either with antennae extended or folded, and the rate of fall depended to a large extent on the position of the antennae. Although a fairly constant ratio was found to exist between the rate of fall with antennae folded and with antennae extended, it was considered more convenient to keep a record only of forms falling with antennae folded, as far as this was possible. It is interesting to note that in this respect *D. pulex* differs from some other plankton Crustacea. G. H. Parker⁽¹⁾ investigated the rate of fall of etherised specimens of the copepod *Labidocera aestiva*, and observed that the antennae are folded in dead forms and extended in narcotised ones.

The dimensions of the animals were found by means of a scale fixed in the eyepiece of a microscope. The breadth was found to bear a fairly constant ratio to the length, so only the length of the animal without the spine was recorded.

A few preliminary experiments were done to determine whether there was sufficient change in the density of the animal from time to time to be perceptible by the method planned. A record of one of these will be given, by which the daily change in the specific gravity of individuals under experimental conditions was determined.

Four individuals, A, B, C, and D, were isolated and fed during the day, and starved and kept in the dark during the night. Their rate of fall was taken night and morning, and the temperature of the vertical tube of water. This was continued for six days; then it was found that curves of rate of fall followed in general direction the temperature curve, so hereafter the temperature was kept constant.

The chief difficulty encountered here was that one individual did not by any means become narcotised always with the antennae in the same position.

For each animal in some cases values were obtained for antennae both extended and folded, so that in other cases when only the "extended" value was known by experiment, the corresponding "folded" value could be calculated.

This will be made clear by the results (Table I), where

1e, 1f represents 1 antenna extended, 1 folded.

2f , 2 antennae folded.

2e , 2 antennae extended.

2f calc. , calculated value with 2 antennae folded, and calculated values are shown in italics.

Where no explanatory mark is given, both antennae were folded.

The results given in Table I are set out graphically in Fig. 1.

The chief points to be noted are:

(1) That there is a marked difference in specific gravity in *D. pulex* after approximately 12 hours feeding or 12 hours starvation.

(2) That the specific gravity of the individual is greatly affected by the condition of the brood pouch; emptying of the brood pouch causes a sudden temporary decrease in specific gravity.

These preliminary experiments which were performed all showed that in *D. pulex* feeding is accompanied by a change in specific gravity great enough to be indicated by the method used.

It is possible that owing to its vertical migrations the animal at times passes out of the region of food supply which likewise varies in distribution according to its heliotropism.

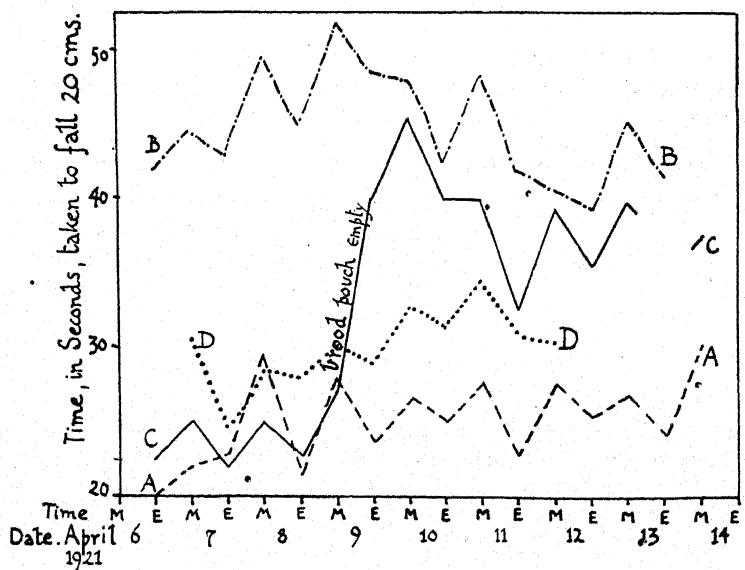
It can be imagined that at dawn, when the light is of moderate intensity, the green algae are strongly positively heliotropic and swarm at the surface. Should the *Daphne* also be at the surface they would feed, and their specific gravity would increase, and possibly they would sink to a depth where the change in temperature and consequently in viscosity and density would bring the energy equation to its right value.

Table I
Daily Variations in Specific Gravity.

Length in millimetres		A 2.32	B 1.40	C 2.00	D 2.05
Date	Temp. °C.				
			Times of fall (secs. for 20 cms.)		
Ap. 6, 7	14.2	20.0	42.0	22.4	2e*
Ap. 7, 10	12.1	22.0	44.6	25.0	30.8
5	12.8	22.8	43.2	29.0 (re, if) 22.0 (2f calc.)	24.0
Ap. 8, 11	10.5	{ 32.2 (re, if) 29.6 (2f calc.)	{ 68.0 (2e) 49.6 (2f calc.)	{ 36.6 (re, if) 27.8 (2f calc.)	29.0
5	11.1	{ 27.0 (re, if) 21.6 (2f calc.)	44.8	22.8	28.0
Ap. 9, 10	10.6	{ 33.4 (re, if) 28.1 (2f calc.)	{ 71.2 (2e) 51.9 (2f calc.)	27.2†	31.2
6	10.8	23.8	{ 67.0 (2e) 48.8 (2f calc.)	39.9	29.0
Ap. 10, 10.15	10.8	26.7	{ 55.4 (re, if) 48.0 (2f)	45.6	{ 33.0 (2f) 44.0 (re, if)
7	11.5	{ 31.2 (re, if) 24.9 (2f)	42.4	40.4	{ 31.4 (2f) 41.6 (re, if)
Ap. 11, 10.45	11.2	{ 33.0 (re, if) 42.0 (2e)	{ 48.1 (2f) 66.0 (2e)	{ 39.8 (2f) 52.4 (re, if)	34.4
5.15 p.m.	12.9	22.8	39.4	32.8	30.1
The temperature was now kept constant at 12.9° C.					
Ap. 12, 10.45	a.m.	—	27.5	40.5	39.4
7	p.m.	—	25.6	{ 45.8 (re, if) 39.7 (2f calc.)	35.6
Ap. 13, 9.45	a.m.	—	26.8	{ 52.6 (re, if) 45.6 (2f calc.)	{ 52.4 (re, if) 39.8 (2f)
8.30 p.m.	—	{ 31.0 (re, if) 24.3 (2f calc.)	{ 57.2 (2e) 47.7 (2f calc.)	54.4 (2e)	—
Ap. 14, 10	a.m.	—	30.5†	47.5 (re, if)	37.3

* Time not taken.

† Brood pouch emptied.



If they were now carried out of the region of food supply, further activity would be accompanied by a gradual decrease in specific gravity.

It was thought that if specimens of *D. pulex* were collected from the same pond at different hours of the day, and their specific gravities compared, some indication might be given of the time of feeding of the animals.

An experiment was arranged to last over twenty-four hours; every two hours a number of *Daphne* were removed from a pond on Sheeps' Green, and at once placed in 1 per cent. urethane. These were then brought back to the laboratory and their rates of fall taken and plotted against their size, twelve curves in this way being obtained.

The greater number of the animals used were in size between 2.5-3.0 mm. and between 1.5-2.0 mm. The average change of velocity for each of these groups during twenty-four hours was determined, and is given in Table II.

These results are shown graphically in Fig. 2. For both these groups it appears that the time of the greatest velocity, and therefore the greatest specific gravity, is about 8 a.m. However there were not enough animals in each group to give conclusive results without further confirmation.

Table II

Time (summer time) 1921	Average velocity Length of animal 1.5-2.0 mm. cms. per sec.	Average velocity Length of animal 2.5-3.0 mm. cms. per sec.
Ap. 20. 6 a.m.	.43	.93
8 "	.61	.98
10 "	.57	.94
12 midday	.57	.94
2 p.m.	.59	.91
4 "	.54	.92
6 "	.51	.88
8 "	.53	.91
10 "	.44	.86
12 midnight	.43	.84
Ap. 21. 2 a.m.	.45	.81
4 "	.43	.80

Only 1 specimen

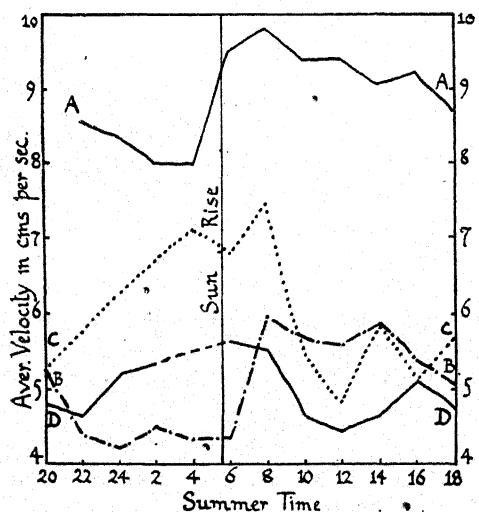


Fig. 2. Changes in Specific Gravity of Collections of *Daphne pulex* during 24 hours.

- Individuals collected 20-21 April 1921, length from 2.5-3.0 mm.
- Individuals collected 20-21 April 1921, length from 1.5-2.0 mm.
- Individuals collected 15-16 August 1922, length from 1.8-2.0 mm.
- Individuals collected 20-21 August 1922, length from 1.6-1.8 mm.

It was planned to repeat the experiment, using only animals of a limited range of size, but this had to be postponed until August, 1922, owing to the entire lack of material.

In August, 1922, two such determinations were made, showing the change of velocity in narcotised specimens of *D. pulex* during twenty-four hours, and the results are shown in Table III. The specimens were obtained from the pond in the grounds of Girton College, Cambridge, by means of a sweeping-net, and were always taken from the same point, where the water was about 18 inches deep. The water became deeper towards the middle of the pond.

Table III

Time (summer time)	Experiment of Aug. 15-16, 1922 Length of animals 1.6-1.8 mm. Temperature constant at 18° C.		Experiment of Aug. 20-21, 1922 Length of animals 1.8-2.0 mm. Temperature constant at 18° C.	
	Remarks on weather, material, etc.	Average velocity for approx. 20 individuals	Remarks on weather, material, etc.	Average velocity for approx. 20 individuals
10 p.m.	Dark	.57	Not quite dark	.47
12 midnight	Material scarce	.63	Material abundant	.52
2 a.m.	Moon just rising	.67	Dark; no moon	.54
	Material very scarce		Material scarce	
	Moon not shining on water		Dark; no moon	
4 "	Material very scarce	.71	Material very scarce	
	Moon not on water		Dark; mist rising	
	Material scarce		Material very scarce indeed	
6 "	Sun rising	.68	Light, very misty	.57
8 "	Material very plentiful	.75	Material very abundant	
	Cloudy		Sun shining, not on water	
	Material abundant		Material less abundant	
10 "	Cloudy	.56	Sky overcast	
	Material less abundant		Material much less plen- tiful	
12 midday	Sun shining, but not on dipping place	.48	Sun not on water	.44
	Material less abundant		Material as at 10 a.m.	
2 p.m.	Sun shining on dipping place	.58	Sun not on water	.46
	Material very scarce		Material as previously	
4 "	Sun not on water	.51	Sun not on water	.51
6 "	Material plentiful	.57	Material as previously	
	Cloudy		Cloudy	
	Material abundant		Material abundant	
8 "	Dull	.52	Dull	.48
	Material very abundant		Material very abundant	

These results are shown graphically in Fig. 2.

The general results of these experiments may be summarised thus;

- (1) All four observations show a maximum specific gravity for *D. pulex* at about 6-8 a.m.
- (2) The two later experiments show a less well-defined minimum specific gravity at about midday.

- (3) *D. pulex* is most abundant in shallow water (18 inches) just about sunrise.
- (4) *D. pulex* is most scarce in shallow water from midnight until after 4 a.m., and when the sun is shining directly on the water.

DISCUSSION.

As *D. pulex* is found in greatest abundance in the shallow margin of the pond just at sunrise, and is most scarce in this water two hours before sunrise, it is obvious that it must come to the surface waters in greatest abundance in the hour before sunrise. This confirms the general conclusion arrived at by C. Juday in his observations on the diurnal movements of plankton Crustacea. The phytoplankton is found most abundantly near the surface: therefore about sunrise *D. pulex* is more completely surrounded by food, and obviously this is the time when it feeds, as it has been shown to attain its maximum specific gravity shortly after sunrise.

It seems reasonable to suppose that the increase in specific gravity of *D. pulex* shortly after sunrise causes it to sink to some point where the temperature of the water is such as to keep constant the right-hand side of the hypothetical equation.

This purely mechanical equation is complicated by the "tropisms" of the animal which would cause it entirely to desert the region of shallow water under the influence of strong sunlight, as was found at 2 p.m. on August 16th, 1922.

SUMMARY.

- (1) It is established that the specific gravity of *Daphne pulex* varies. The state of starvation of the animal, and the number of young in the brood pouch, separately affect the specific gravity.
- (2) The release of the young from the brood pouch causes an instantaneous decrease of specific gravity.
- (3) The specific gravity varies in animals of the same size at different periods of the day and night, attaining a maximum shortly after sunrise, and reaching a less well-defined minimum about midday.
- (4) *D. pulex* is most abundant in shallow water shortly before sunrise, and most scarce from midnight till just before dawn, and when the sun is shining directly on the water.
- (5) Changes in specific gravity must be taken into consideration when investigating the causes of the vertical distribution of the plankton.

My thanks are due to Mr J. T. Saunders, who suggested to me that I should investigate this subject and assisted me during the course of the work. He is responsible for the records during the night of 20/21 April, 1921.

REFERENCES.

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JUDAY, C. (1904). "The diurnal movements of plankton Crustacea." *Trans. Wis. Acad. Sci. Arts and Lit.* 14, 534-68.

ON THE INVASION OF WOODY TISSUES BY WOUND PARASITES

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AND W. C. MOORE, B.A.

Major Scholar, Trinity College, Cambridge.

(With One Text-figure.)

WHILE investigating the biology of wound parasitism as shown by *Stereum purpureum*—the fungus which causes Silver-leaf disease of fruit trees—some interesting facts have recently come to hand concerning the initiation of invasion by this organism. Hitherto little attention has been paid to the exact manner in which wound parasites invade their hosts, especially those like *Stereum purpureum* which infect woody tissues rather than the bark. Recent papers on Silver-leaf disease* have shown that this fungus indubitably behaves as a wound parasite, *i.e.* that infection of a plum tree for instance only occurs when spores alight upon some wound and there germinate, forming a mycelium which passes deep into the tissues. It was formerly supposed that the spores of such a fungus germinated on the surface of the wound, producing germ tubes which in some way or other passed into the wood, although it was difficult to understand how the young hyphae were orientated into the vessels. In a further paper on Silver-leaf disease†, which is in the press, it is shown that at certain times of the year the spores of *Stereum purpureum* will readily infect the freshly cut extremities of plum shoots kept in water, passing rapidly thence through the woody parts and killing the tissues as it goes. In view of the ease with which inoculation experiments of this kind can be carried out both in the laboratory and in the field, it was thought worth while to examine carefully the initiation of infection. In the first experiments, three-year-old shoots of Victoria plum trees, about 8 inches long, were placed in water in the laboratory, and upon their freshly cut upper extremities an emulsion of spores of *Stereum purpureum* in sterile water was applied. The shoots were then left exposed to the atmosphere, and longitudinal sections were cut through the inoculated ends of the twigs at intervals of two, four, and six days after the spores had been added. Sections cut after a period of two days showed that the majority of the spores had been taken into the vessels, some to a distance of 3 mm., where they were beginning to germinate. A few spores were left on the surface of the wood but these were not

* Brooks, F. T. "Silver-leaf disease, I and II," *Journ. Agric. Sci.* 1911 and 1913. Brooks, F. T. and Bailey, M. A. "Silver-leaf disease, III," *Ibid.* 1919.

† Brooks, F. T. and Storey, H. H. "Silver-leaf disease, IV," *Journal of Pomology and Horticultural Science*, 1923.

germinating, and no spores had been sucked into the other cells of the wood, the pith, or the bark. It should be stated here that the spores of *Stereum purpureum* are very minute, their longest axis being only about one-sixth the diameter of plum wood vessels. Sections of inoculated extremities cut after four and six days indicated that the spores had formed a vigorous mycelium which was quickly passing down into the wood through the vessels. In sections cut after an interval of four days a few spores on the surface were found to be germinating, but these could play no part in infection as the wood had already been invaded to a distance of 20 mm.

The next experiment was framed to show the position of the spores immediately after the emulsion had been applied. As soon as possible, therefore, after spores had been added to a fresh surface, sections were cut, when it was found that the spores had been likewise taken up into the vessels, this time to a maximum distance of 6 mm.

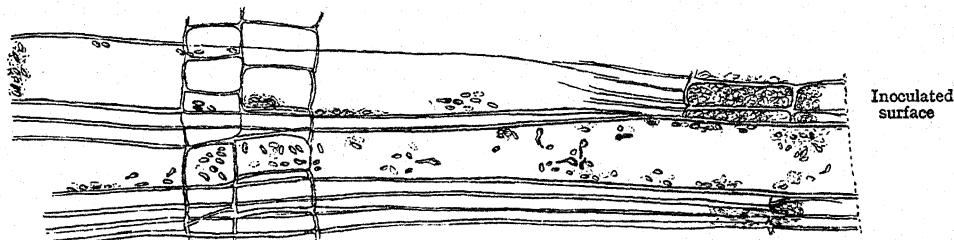


Fig. 1. Section of an inoculated plum shoot showing spores of *S. purpureum* carried into and germinating in the vessels. $\times 100$.

March 12: surface inoculated with dry spores.
 March 13: rain fell heavily.
 March 14: shoot cut off and taken to laboratory.
 March 15: sections cut and stained with lactophenol-cotton blue.

It having been demonstrated that most of the spores were sucked into the vessels instead of remaining on the surface, Indian ink was similarly applied to freshly cut extremities of plum shoots to ascertain whether small inert particles were also carried downwards. Sections showed that the particles suspended in Indian ink were also immediately taken into the vessels, frequently farther than the spores. It is clear, therefore, that the sucking of the spores into the vessels is a purely passive process as far as the spores are concerned, but one which, as will be explained, may be advantageous to the fungus.

Changes were then made in the mode of application of the spores to the cut wood to try to determine the range of conditions under which the spores were taken into the water-conducting tracts. Spores added in the dry state were not taken into the vessels except occasionally to a distance of 1 mm. Few of these spores germinated whether on the surface or just within the vessels. Spores were also applied to surfaces which had been cut one or several days previously, and, when an emulsion was used, always with the result that the spores were taken a considerable distance into the vessels, although not usually so far as with newly exposed tissues.

It was important to ascertain whether the phenomena of infection were the same under natural conditions, so spores were applied in the same fashion to wounds

made on fruit trees in the open. The results were found to be the same as those in the laboratory. It is safe to conclude, therefore, that for the time of year when these experiments were made, viz. the autumn and winter, the carrying of the spores of this fungus into the vessels before germination is of general occurrence.

Fig. 1 is a drawing of a section of a plum shoot inoculated in the field showing the spores deep in the vessels and beginning to germinate. In this experiment the spores were carried into the vessels owing to the fall of rain a day after the spores were applied in the dry state.

The physical forces which carry the spores into the vessels may be of diverse kinds according to the conditions under which the spores are placed on the surface. In a freshly cut branch, exposure to the atmosphere probably causes a depression of the water-level in the vessels, which results in the immediate absorption of the spore emulsion by these capillaries. Subsequent desiccation at the surface may increase the distance to which the spores are taken into the vessels. On the other hand, with wood which has been allowed to dry before the spore emulsion is added, the ends of the capillaries would immediately absorb the spores with the fluid.

It is likely that the carrying of spores into the vessels before germination will be found to be of general occurrence in wound parasites which invade woody tissues; further enquiries are being made about this. Likewise experiments with *Stereum purpureum* are being continued to see whether the phenomena of infection are the same all the year round.

The advantage to such a fungus of its spores being taken into the vessels is twofold: the delicate germ tubes are protected from periods of desiccation to which they would often be subject on the surface of the wood, and, secondly, under these conditions, the germ tubes arise actually in the cells which are the main channels of invasion in this class of parasite. It is of interest that the spores of other wound parasites which infect through the wood rather than the bark, e.g. *Polyporus squamosus*, *Collybia velutipes*, etc., are universally small, so that they can be readily taken into the vessels in the manner described.

ON THE STRUCTURE OF A MIDDLE CAMBRIAN
ALGA FROM BRITISH COLUMBIA (*MARPOLIA*
SPISSA WALCOTT)

By J. WALTON, M.A.

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(With One Text-figure and Plate V.)

IN 1919 Professor Walcott* described some fossil algae from the Burgess Shales (Middle Cambrian), British Columbia. Among these some show features which he considers afford evidence of a relationship to the *Nostocaceae*, a family of the *Myxophyceae* or Blue-green Algae. Recently some specimens of these fossils were acquired by the Sedgwick Museum, Cambridge, and thanks to the kindness of Mr King, the Curator, a few fragments were lent to me for purposes of investigation. By a process in which the organic remains exposed on the surface of the rock are transferred to a transparent base of fused balsam† it is possible to examine the isolated fossil by transmitted light with high powers of the microscope. A photograph of a transfer-preparation of one of these algae, *Marpolia spissa* Walcott, is given in Pl. V, figs. 1, 2. Several preparations were made and on examination of these no regular septation such as is described by Prof. Walcott‡ was observed. It is possible that the appearance presented by the filaments when observed by reflected light on the rock surface led Prof. Walcott to interpret the structure observed as evidence of septate filaments such as are found in the *Cladophoraceae*. The branching is irregular; one of the few cases observed is figured in Text-fig. 1.

The filaments (9-40 μ in diameter) are broken up by transverse cracks (the transverse lines described by Prof. Walcott) into separate portions, an effect due to some purely physical action comparable in its result to the forces that produce the "cleat" in coal and in other carbonaceous incrustations. When the rock was split open the residual organic material was shared by both surfaces and thus only an incomplete fossil is seen on examining either surface separately. The filaments are frequently found twisted together forming a loose cable-strand structure. When examined by reflected light they are seen to be finely ridged longitudinally. The examination by transmitted light corroborates this observation, for a great many of the filaments are partly translucent and darker stripes (diameter 4 μ) of denser substance are visible running longitudinally in the filament (Pl. V, fig. 3). In some cases the stripes can be followed over considerable lengths of a single filament and must represent some feature in the structure of the original organism.

* Walcott, C. D. *Smithsonian Miscellaneous Collections*, 67, No. 5.

† Walton, J. *Annals of Botany*, 1923. In the press. ‡ Walcott, C. D. *Loc. cit.* p. 234, l. 15.

Sometimes a more complicated structure is found, replacing the longitudinal stripes, consisting of a series of short transverse bands inside the translucent portion of the filaments (Pl. V, figs. 4 and 4 a). The distance between these bands, when only a single series is represented, is about 3μ . Frequently entirely translucent filaments are found and I regard these as the sheaths or portions of the sheaths which surrounded what were originally trichome-like structures now represented in the fossil by the dark stripes. The actual cellular constitution of the stripes or trichomes is conceivably expressed in places by the appearance of the short transverse bands which may themselves represent the contracted contents of disc-shaped cells similar to those described in *Archaeothrix oscillatoriformis** Kidston and Lang, an alga attributed to the *Schizophyceae* (*Myxophyceae*), from the Middle Old Red Sandstone of Scotland or in the modern *Oscillatoria*. The difference in optical density of the sheath and the stripe represents the difference which would be found in algal filaments with stout mucilaginous sheath such as



Fig. 1. *Marpolia spissa* Walcott. $\times 65$.

occur in some of the *Myxophyceae* at the present day. There is quite enough organic material in such sheaths to give a black carbonaceous film in the fossil state. It must be noticed, too, in support of this contention, that soft-bodied animals which could not have had a much greater dry-weight than jellyfish are very perfectly preserved in the Burgess Shales. Gomont† states that the cell wall in the *Nostocaceae* is like cutin in composition while the sheath is more nearly akin to cellulose. If these algae are subjected to greater exposure to the atmosphere and greater illumination than that to which they are usually subject, the sheath changes in composition and becomes more or less cutinised‡. This seems additional evidence in favour of the possibility that a sheath of this nature may be preserved in these Cambrian fossils, for we know that at least in Devonian plants cuticularised membranes are preserved in a modified condition in sandstone§.

* Kidston and Lang, *Trans. Roy. Soc. Edin.* 52, Part IV (No. 33), p. 875.

† Gomont, "Monographie des Oscillariées," *Ann. Sci. Nat. Bot. Ser. VII*, 15, p. 282, 1. 9.

‡ Gomont. *Loc. cit.* p. 282, 1. 30.

§ Walton, J. *Annals of Botany*, 1923. In the press.

A portion of one of the translucent filaments was seen with somewhat irregularly placed transverse bands which apparently projected slightly at the sides of the filaments (Pl. V, fig. 5). This may be interpreted as a wrinkling of the gelatinous sheath, the dark bands representing a fold where possibly three thicknesses of the sheath are superposed.

COMPARISON WITH LIVING FORMS.

The suggestion of a sheath-like structure enclosing darker strands at once suggested a comparison with certain genera of the *Myxophyceae* in which several filaments of the *Oscillatoria* type are found united in a gelatinous sheath. The general habit (Text-fig. 1) agrees closely with what is found in the genus *Schizothrix** Kützing, in which the sheath is very cartilaginous. The short transverse bands found in regular series may be interpreted as the remains of cellular structure in individual trichomes. As far as dimensions are concerned the correspondence between the fossil and the *Myxophyceae* is fairly close. The dimensions of *Marpolia* fall within the limits found in the genus *Schizothrix*† and there can be little objection to a comparison on such grounds. Thus there are several small points which though insignificant taken separately, together afford considerable evidence for supposing that *Marpolia* has the same general structure as *Schizothrix*:

1. Habit like that of *Schizothrix*.
2. Indications of several trichome-like structures inside a sheath of less solid material.
3. Evidence of cellular structure in the trichome-like structures.
4. Wrinklings in sheath.
5. Correspondence in dimensions.

It would be interesting to know why Prof. Walcott assigns this organism of such enormous antiquity to the comparatively narrow limits of one of the families of the modern *Myxophyceae*. Indeed he compares its structure to that of the *Cladophoraceae*, a group absolutely distinct structurally and phylogenetically from the *Myxophyceae*. However, if the evidence brought forward in this note is considered, it is clear that if this plant is to be assigned to any group in existence at the present day it must be to the latter group. At present, however, the available information about the structure of the internal strands is not sufficiently complete to warrant our assuming with certainty that the internal strands were similar in organisation to the trichomes of *Oscillatoria* and although the general structure is the same as that of *Schizothrix* it seems that it is better to place it provisionally in the *Protophyceae*‡, a non-committal term for primitive algae whose relationships to the modern phyla are unknown or uncertain. I think that it is highly probable that investigation, on the above lines, of more material would yield further information about this extremely early algal organism which may now be claimed as the earliest plant exhibiting internal organisation.

* Gomont. *Loc. cit.* p. 293 and pls. VIII, IX, X.

† Tilden, J. "Minnesota Algae." Vol. I. *Report of the Survey Botanical Series*, 1910, p. 150.

‡ Lindenbein, H. (1921). "Les Protophycées, etc." *Bull. Soc. Bot. Genève*, p. 289.

DESCRIPTION OF PLATE V.

The photographs, taken from transfer-preparations, are by the Author.

Marpolia spissa Walcott.

FIG. 1. Tangled strands of the alga, the individual filaments of which show considerable variation in diameter. $\times 20$.

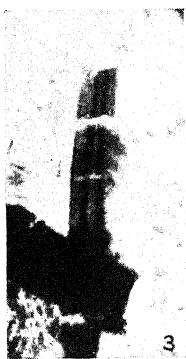
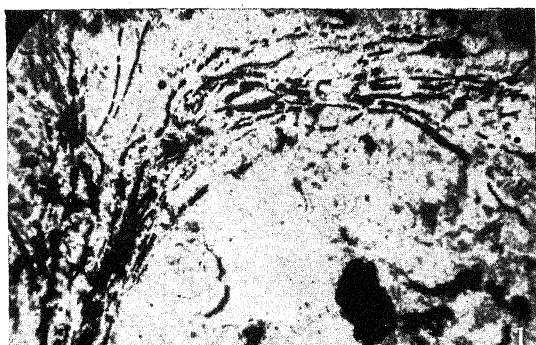
FIG. 2. An example of the aggregation of the filaments into close bundles. $\times 20$.

FIG. 3. Portion of partially translucent filament with two dark stripes running longitudinally within it. The filament exhibits several transverse cracks. $\times 300$.

FIG. 4. Portions of filaments at greater magnification. $\times 350$.

FIG. 4 a. Enlargement from Fig. 4 of portions of filaments exhibiting internal structure expressed by the series of short transverse bands seen in the filament near the centre of the figure below *a*. Two isolated bands may be seen to the right of *b*. $\times 500$.

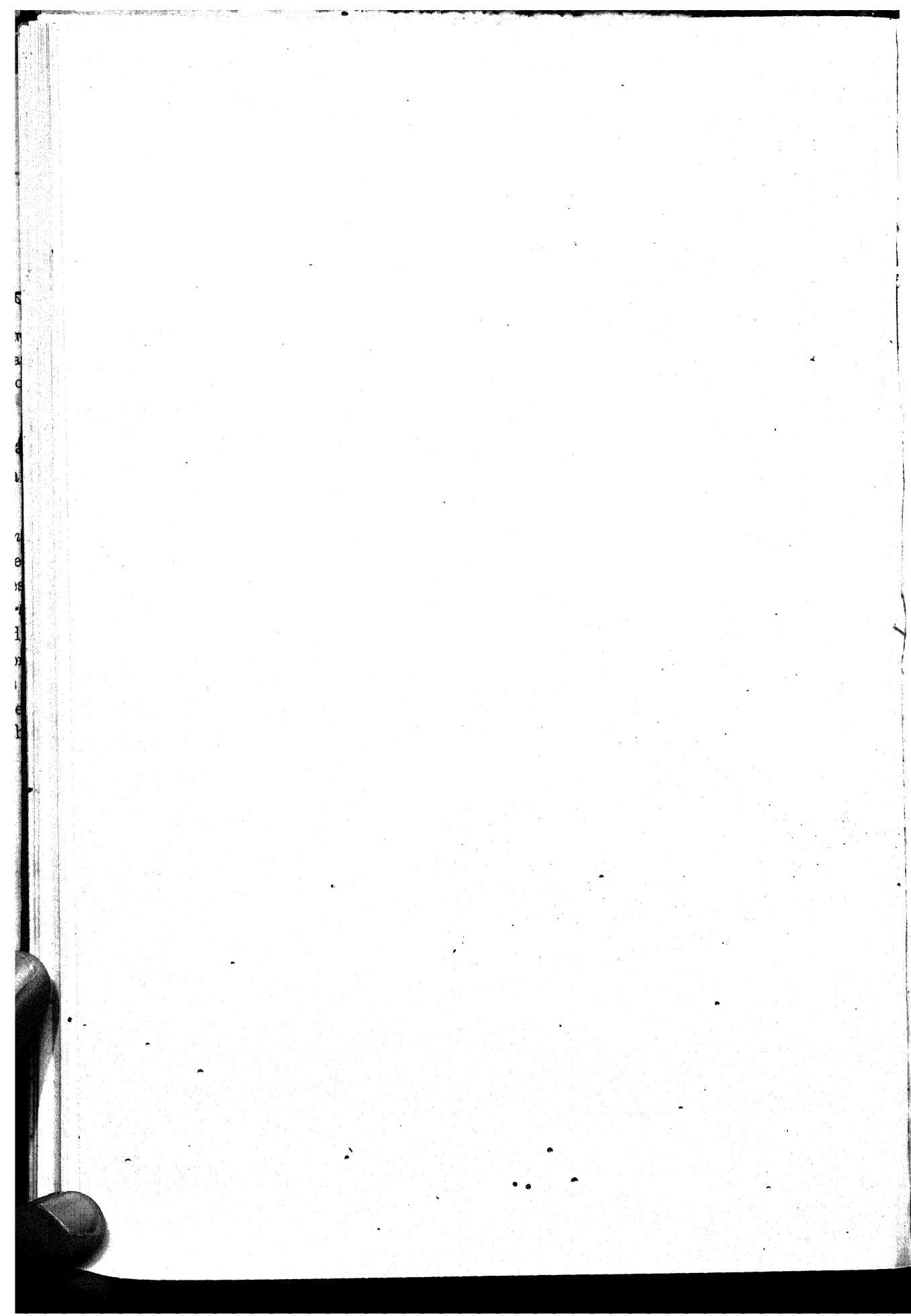
FIG. 5. Filament with transverse wrinkles in the sheath at *c*. $\times 500$.



Phot. J. W.

Marpolia spissa Walcott.

WALTON—STRUCTURE OF A MIDDLE CAMBRIAN ALGA (pp. 59-62).



ON THE APPEARANCE OF GAS IN THE TRACHEAE OF INSECTS

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(Received 7 February 1924.)

(With Three Text-figures.)

REVIEW OF PREVIOUS WORK.

IT is well known that the tracheae of insects before, and often for a short period after, their hatching from the egg are filled with a fluid, which suddenly disappears and is replaced by gas. A similar phenomenon takes place at each moult and occurs, not only in terrestrial insects provided with normal spiracles, but also in aquatic insects, which are devoid of functional spiracles.

This fact was noted for the first time by Meinert (1886) in his physiological study of *Corethra* larvae, which are apneustic (devoid of functional spiracles) and are provided with four air sacs. He found that the newly hatched larvae have their air sacs filled with a "serum," but that soon after hatching these sacs suddenly become filled with air.

The great difficulty in explaining this phenomenon, especially in aquatic insects devoid of spiracles, was clearly pointed out by Miall (1895, p. 37) in his *Natural History of Aquatic Insects*. Sadones (1895), in his study on the respiratory gills in Dragon-fly larvae, arrived at the conclusion that the diffusion theory cannot explain the first appearance of gas in the tracheae. He goes even further by saying that not only the appearance of the gas but the ultimate respiratory exchange in these insects can only be explained by a special secretory function of the living protoplasm. Calvert (1898) was the first actually to observe the appearance of air in the tracheal system of young dragon-fly larvae (*Sympetrum vicinum*). He found that the gas appears suddenly in the tracheal trunk and thence rapidly spreads forwards and backwards into the capillaries.

Krogh (1911), while working on the hydrostatic mechanism in *Corethra* larvae, found that the air sacs in a full-grown larva submitted to a high pressure (70-80 cm.) become filled with fluid and usually die in a few days. He records however a single case of the partial absorption of the fluid from the air sac, with the reappearance of air. He has shown also that in *Corethra* larvae "gas secretion does not take place at all" and that the hydrostatic function of the bladders of these larvae is governed by the secretion of the fluid. Krogh compares the bladders to the ballast tanks of a submarine boat and continues: "If the animal becomes too heavy, water is pumped out of them and if it becomes too light it is pumped in until equilibrium with the water is restored." There is no indication, however,

in Krogh's paper as to the mechanism of this fluid secretion or as to the cells which may perform this function.

Winterstein (1912), who strongly supports the physical explanation of the respiratory gas exchanges in all animals, admits however that in the case of aquatic insects the diffusion theory does not explain the initial filling of tracheae with gas. This phenomenon, he thinks, can be understood only by postulating the secretory activity of the living cells.

More recently von Frankenberg (1915) has reinvestigated the problem of the appearance of the gas in the tracheae of *Corethra* larvae and the following are the main results of his observations:

(1) The process of filling with gas is very rapid, almost sudden, and takes place about five minutes after the hatching of the larva from the egg.

(2) The gas appears in the posterior end of the tracheal trunks, just behind the posterior air sacs and spreads rapidly forward filling both pairs of air sacs, the two lateral tracheal trunks and the transverse tracheal branches. It then rapidly disappears from the tracheae, remaining only in the four air sacs.

The gas does not appear when the larvae are previously kept in boiled water devoid of air. The variation in pressure has no effect upon the appearance of gas in the tracheae.

In a larva with the head amputated the four sacs become normally filled with gas. In a larva cut in half the posterior sacs only are filled with gas and finally, when the posterior end of a larva is cut off just behind the posterior air sacs, the gas does not appear at all.

According to von Frankenberg these observations and experiments indicate that the gas which appears in the sacs of the *Corethra* larva is secreted in the 11th segment of the body.

The general conclusions of von Frankenberg have been confirmed by Pause (1919) on the larvae of *Chironomus*, the tracheal system of which is very incomplete and can be seen only in the anterior segments of the body. In such larvae the air seems to appear in the peripheral capillaries and thence spreads towards the larger tracheae.

Akenhurst (1922) arrived at a similar conclusion in his study of *Corethra* larva.

Pause thinks that the appearance of the gas in the tracheal system requires a considerable amount of energy, which is used: (1) for overcoming the capillary resistance of the fluid column filling the tracheae and (2) for expelling this fluid through the walls of the tracheae into the body cavity. The initial appearance of the gas would then be due not to a simple osmosis and diffusion but to the secretory activity of the living cells, namely the secretion of gas.

The statements of v. Frankenberg and Pause show that the secretory origin of the gas in the tracheae was accepted by them as the alternative explanation to the diffusion theory. No evidence for the secretory theory has yet appeared however. On the contrary, neither of these authors has been able to reveal the slightest positive evidence for the existence of these gas-secreting cells in the flattened epithelium lining the tracheal tubes, and subsequent study by the present

writer has equally failed to reveal them there or in any other position. Moreover, the secretory theory gives no explanation as to the fate of the fluid which fills the completely closed system of tracheal tubes, the walls of which are not readily permeable to fluids. Finally, the secretion of the gas into the tracheal tubes and diffusion of fluid out of them cannot possibly take place with such remarkable synchronism and rapidity as characterises the filling of the tracheae with gas. The secretory explanation of this phenomenon can therefore be rejected as not being supported by evidence.

The problem of the appearance of gas in the tracheae has been dealt with on several occasions by Tillyard (1915-1916) in the course of his investigations on Dragon-flies. His observations on the newly-hatched larvae of *Anax papuensis* confirm those of Calvert. In addition, Tillyard believes that he has also found the real explanation of this phenomenon, an explanation based solely upon the diffusion theory of gases. The following is the evidence he puts forward in support of his view:

(1) The first and most important fact is that the gas appears in the main tracheal trunks and thence spreads towards the capillaries.

(2) To discover the nature of the phenomenon he performed a few experiments, two of which, considered by him as conclusive, were:

(a) Two larvae, 1 to 2 days old, put in 10% solution of KOH died about an hour later; but the tracheal trunks and the capillaries of the rectal gills remained filled with air.

(b) Two larvae, less than two hours old, put in a 10% solution of KOH died in about $\frac{1}{2}$ hour; $\frac{3}{4}$ to 1 hour later, the tracheal trunks began to collapse. After two hours all the abdominal tracheae collapsed except the capillaries of the gills, some of which remained intact even after three hours.

From these and other similar experiments Tillyard concluded that the gas which appears in the tracheal trunks must be CO_2 and that it appears as a result of increased work performed by the larva during the process of hatching from the egg or the pronymphal cuticle.

As to the fluid filling the tracheae, which he assumes to be blood, he writes that "it passes into the haemocoel and the connection between the dorsal tracheae and the aorta must be sought for in the cephalic heart."

One useful fact emerges from Tillyard's observations, which is that the gas appears first in the tracheal trunks and not in the capillaries. His assumptions that the fluid in the trachea is blood and that a possible connection may exist between the tracheal trunks and the aorta are completely unjustified, as it is a well established fact for all the Tracheates that the tracheal system does not communicate with the body cavity. As to the fluid which fills the new tracheae at each moult, it is undoubtedly similar to the ecdysial fluid which is secreted by the hypodermal cells and which separates the two cuticular layers of the insect. Experiments in putting fragile insects into a 10% solution of KOH are too crude to give the slightest indication as to the presence or absence of CO_2 in the tracheal trunks. Under ordinary conditions the large trunks of dead insects some-

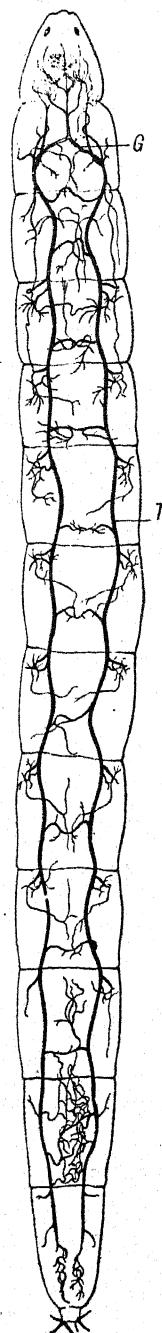


FIG. 1. *Dasyhelea obscura*. Larva seen dorsally, showing the tracheal trunks (T) with the main tracheal branches and the place of the first appearance of gas (G).

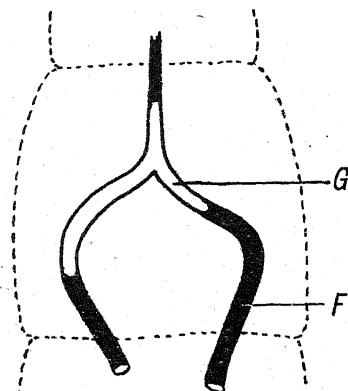


FIG. 2. The anterior portion of the tracheal system under higher magnification showing the appearance of gas (G); F—tracheal fluid.

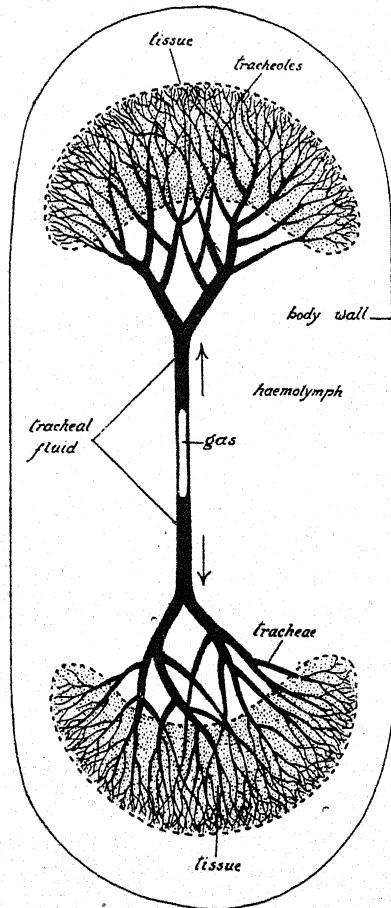


FIG. 3. Schematic figure representing the mechanism of the filling of tracheae with gas.

times become filled with fluid or even collapse, in many cases before the small branches or the capillaries. But in most cases, while the animal remains alive, and often even after death, the tracheal trunks resist a pressure (positive or negative) of about 1 atmosphere. The collapse of the tracheal trunks in Tillyard's experiments is not due therefore to the absorption of CO_2 but to the action of KOH upon the walls of the tracheae.

Moreover, if it could be proved that the gas appearing in the tracheal trunks was pure CO_2 , it would be impossible to explain its origin otherwise than by accepting the theory of the secretory activity of the living cells. Indeed, we know that CO_2 is a gas 30 times more soluble than O_2 and that it easily enters into various combinations in the tissues and blood of the animal. It is therefore easy to discover what becomes of CO_2 during and after the effort of hatching. To appear in the form of a gas the tissues and blood of the animal must reach such a state of saturation with CO_2 as can be found only in mineral waters. It will be seen then that neither v. Frankenberg's and Pause's secretory- nor Tillyard's diffusion-theories explain the mechanism of the appearance of gas in the aquatic apneustic insects.

PERSONAL OBSERVATIONS AND A NEW EXPLANATION OF THE PHENOMENON.

I have studied this phenomenon in the larvae of a midge, *Dasyhelea obscura* Winn. (Ceratopogoninae), commonly found in the fluid, decomposed sap filling the wounds of various trees. These larvae, like those of all other Chironomids, are apneustic (*i.e.* completely devoid of functional spiracles) though they have a very well-developed tracheal system filled with air, in all respects similar to that of larvae provided with open spiracles. Immediately after hatching from the egg, and also after each moult, the tracheal system of the *Dasyhelea* larva is always filled with a fluid. When these larvae, mounted in a small cell filled with water, are carefully watched under the microscope the gas may be seen to appear suddenly in the anterior portion of the main tracheal trunk (Figs. 1 and 2) and to spread thence forwards and backwards into the secondary branches, finally reaching the capillary tracheoles which ramify within the various tissues. The complete filling of the whole tracheal system with gas is usually accomplished in less than one minute. The columns of tracheal fluid, once ruptured, rapidly but evenly move in opposite directions, passing from the tracheal trunks towards the capillaries and giving place to the gas which steadily spreads and fills the whole tracheal system. Observation of this phenomenon suggests the possibility of a new and simple explanation of its mechanism which, in opposition to the previous theories, accounts not only for the appearance of gas, but also for the fate of the tracheal fluid. The tracheal system of an apneustic larva can be represented schematically in the form of a rigid tube, giving rise at each end to a series of ramified branches, which eventually form two large arborescent systems of capillary tracheoles (Fig. 3). The latter penetrate into the various tissues and finally ramify within the protoplasm of the cells. The terminal intraprotoplasmic branches are very thin and hardly perceptible, those for instance found by R. Cajal (1890)

being only of 0.2μ in diameter. Bearing in mind that the whole system is completely closed and is filled with a fluid which has no communication with the surrounding haemolymph of the body cavity, we must suppose now that the protoplasm of the cells suddenly takes up the fluid filling the terminal intracellular tracheoles, the tissues thus acting as two very strong absorbent systems for the elimination of the tracheal fluid. As a result of this absorption two eventualities may arise: (1) if the small portion of the tracheae where the air appears could not resist the pressure of about one atmosphere, it would collapse without the appearance of gas, but (2) if it is able to resist this pressure, as is the case in these insects, the column of the fluid filling the trachea will be ruptured and its two portions will rapidly move towards the tracheoles, *i.e.* towards the place of absorption. The space left by the fluid will be immediately filled with gases diffusing from the blood and expanding behind the retiring columns of fluid until they reach the final branches of the tracheoles*.

The place of rupture of the fluid column varies from one species to another and may be localised into one or two spots, or may occur at many points of the tracheal system, depending on the distribution of the tracheal ramifications.

We can explain now why in v. Frankenberg's experiments, when the body of the larva was cut behind the posterior pair of vesicles, the air did not appear in the tracheal system. By exposing to the water the cut lumen of the tracheae, at the point where the rupture of the fluid column usually takes place, the water is drawn into the system and the gas fails to appear when the tracheal fluid is absorbed by the tissues.

The advantage of our hypothesis consists in that it explains at once the fate of the tracheal fluid as well as the origin of the gas, both being due to one cause—the absorption of the tracheal fluid by the cells of the body. It is not the gas which expels the tracheal fluid but the retiring fluid which leaves the space for the expanding gas. This hypothesis partly corroborates also the view expressed by Krogh, namely that the hydrostatic mechanism in *Corethra* larva is not governed by the *secretion* of the gas but by that of the fluid (cf. p. 1).

It must be kept in mind, however, that great force must be exerted to rupture the fluid column before the appearance of the first bubble of gas. This force must be directed against the considerable cohesive or tensile strength of the fluid which completely fills a system of rigid tracheal tubes†. The absorption of the tracheal fluid by the tissues cannot therefore be considered as a process similar to that of ordinary suction but must be due either to protoplasmic imbibition or to a

* It is interesting to note that the terminal tracheal branches are more permeable to fluids than the rest of the tracheal system. This permeability misled some workers (Wistinghausen (1890) and Holmgren (1896)) who arrived at the conclusion that the terminal tracheal capillaries are always filled with fluid. Pantel (1898) has shown however that in living specimens they are always filled with gas and that the fluid appears only after the death of the insect.

† We know, in fact, from the experiments of Berthelot (1850) and those of Dixon (1909-14), that the force required to rupture a column of fluid completely filling a rigid tube may reach many atmospheres. And this equally applies to fluids containing dissolved gases or devoid of them. It is possible, however, that in our case the adhesion between the fluid and the walls of the trachea is very feeble, so that a much smaller force is required to rupture the fluid column filling the tracheae.

chemical reaction taking place in the cells. When the force of absorption overcomes that of cohesion, the column of fluid becomes ruptured and the first bubble of gas appears. The broken columns of fluid, relieved from their tensile force, are rapidly pulled from the large trachea into the smallest ramifications until they disappear within the protoplasm of the cells.

Finally, in relation to our problem we may recall here a phenomenon with which many entomologists are familiar, namely the accidental appearance of air in *dead specimens* of insects mounted in viscous fluids such as gum arabic, glycerin-jelly or Canada balsam. This accident, which is so annoying to the amateur naturalist, can be used with profit for the study of the tracheal system in preserved specimens of insects. An insect cleared in xylol or toluol and mounted rapidly in thick Canada balsam, may be made to reveal the whole tracheal system, which having previously been filled with the clearing agent, is now invaded by gas. This appearance of air is easily explained by ordinary laws of diffusion. The xylol rapidly diffuses out of the tracheal tubes while the thick Canada balsam does not penetrate into them. A vacuum appears in the latter, but, being resistant to the external atmospheric pressure the tracheae do not collapse and their lumen becomes rapidly filled with gases or vapours diffusing from the surrounding medium.

The same results may be obtained when a preserved larva having the whole of its tracheal system filled with water is rapidly mounted in a thick glycerin-jelly or gum arabic.

SUMMARY.

- (1) The aquatic apneustic insects, on hatching from eggs or after each moult, have their tracheal system filled with fluid.
- (2) A short time after hatching, or moult, the gas suddenly appears, usually in the large tracheal trunks, and thence rapidly spreads into the capillaries.
- (3) There is no evidence of the secretory origin of this gas, the gas-secreting cells having never been revealed in insects.
- (4) The secretory theory of gas does not explain the fate of the disappearing tracheal fluid. Frankenberg's, Pause's and Tillyard's suppositions are discussed and rejected.
- (5) The following new explanation of the appearance of the gas in the tracheae is proposed:—The tracheal fluid is absorbed by the cells of various tissues from the intracellular capillary tracheoles; the column of the fluid is thus ruptured, the space left by the retiring fluid being immediately filled by gases diffusing from the surrounding media (blood).

This supposition explains both the elimination of the tracheal fluid and the appearance of the gas as being due to the same cause—absorption of the tracheal fluid by the tissue cells.

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THE SPAWNING OF ECHINOIDS

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(Read 26 November 1923.)

THE Red Sea echinoid *Centrechinus (Diadema) setosus* has been shown to exhibit a lunar periodicity in reproduction^(1,2). The genital products are shed at each full moon during the breeding season. Investigation of another echinoid (*Strongylocentrotus lividus*) at Alexandria and at Roscoff has demonstrated that in this case there is no lunar period in the reproduction but that the majority of individuals of both sexes spawn simultaneously at irregular intervals⁽²⁾. The reason for this behaviour, periodic or non-periodic, is unknown, but as a first step to elucidating the causes I studied last summer the mechanism and stimuli of spawning in *Strongylocentrotus lividus*. The work was done at the Roscoff Marine Biological Station and my best thanks are due to the staff for providing abundant material and facilities for investigation.

It is well known that a variety of abnormal conditions cause the spawning of ripe urchins in captivity. This frequently happens in confined jars on the return from fishing, after exposure to the air, or before death. But the natural spawning stimuli and mechanism have so far been unknown.

If a ripe urchin is opened by an equatorial cut and the aboral hemisphere reversed so that the genital pores dip beneath water in a beaker, eggs or spermatozoa are extruded. This is a convenient mode of obtaining the genital products cleanly for experimental purposes. Mr J. Gray informed me from the Marine Station, Millport, last summer that gentle pressure of the fingers on the ovaries or testes of *Echinus esculentus* causes a more ready shedding. The pressure is not necessary however for stimulation by stroking the surface of the gonad of *Strongylocentrotus* once with a camel's hair brush induces the extrusion of eggs or sperm. This is not simply due to the pressure of the brush because (1) shedding occurs after the momentary stroking has ceased, and (2) a visible contraction of the ovary takes place. When an urchin is opened and the coelomic fluid poured out the middle lobe of the central ridge of each ovary falls over to one side. Stimulation with the camel's hair brush causes ridge and lobe to be erected, the erection taking about 10 seconds. It occurs in unripe as well as in ripe ovaries. In males the movement of the gonad is small in extent compared with that seen in females. If an individual is ripe the erection initiates the shedding of the eggs or sperm.

Spawning is thus in appearance due to the contraction of muscles in the gonad walls, and that such muscles are indeed present is shown by microscopic sections. The muscle fibres lie immediately below the peritoneum and are much more abundant in the female than in the male. This accords with the observation that the stimulated testis shows less movement than the ovary. It is probable that less muscular effort is necessary to extrude sperm than eggs.

As to the stimuli causing in nature the contraction of these muscles and consequent spawning, one point only has been elucidated, namely, that the extrusion of genital products by one individual causes the spawning of all other ripe individuals in the neighbourhood. Experiments demonstrating this were made in the aquarium tanks at Roscoff in August last, with a collection of *Strongy-*

Table I
Experiment commenced at 2.40 p.m.

Times at which urchins spawned	Numbers of urchins which spawned			
	In tank B (containing sperm)		In tank C (containing eggs)	
	Males	Females	Males	Females
2.50	—	—	4	—
3.0	—	—	—	1
3.4	—	—	1	—
3.5	—	—	1	—
3.23	1	—	—	2
3.25	—	—	—	2
3.28	—	1	—	1
3.37	—	3	—	—
3.38	—	—	—	—
3.39	2	—	—	—
3.40	—	—	—	—
3.41	—	1	—	—
3.43	—	1	—	—
3.44	1	—	—	—
3.45	2	—	—	—
3.53	—	1	—	1
3.58	1	1	—	1
4.4	—	2	—	—
4.12	—	1	—	—
4.17	—	—	—	—
4.25	—	—	—	1

Experiment discontinued 4.25. There remained four urchins without spawning in tank B, three urchins in tank C.

locentrotus luidus almost all of which were ripe. Three tanks were prepared, (A) filled with plain sea water, (B) with water containing spermatozoa, and (C) with water containing eggs freshly spawned. Into each of these tanks 24 urchins were put. In the course of two hours none spawned in the plain water of tank A, while 20 spawned in B and 21 in C. Table I gives the numbers of individuals with their sex which spawned at the times indicated in tanks B and C: Each animal that spawned was immediately removed.

The experiment was repeated with different individuals in other tanks similarly prepared. This time 18 individuals were placed in each tank. Again none spawned in the control tank A containing plain sea water. The details of the spawning in tanks B and C are given by Table II.

A third experiment was then made, 14 fresh urchins being placed in each of three newly prepared tanks. As before none spawned in the control tank A, while 12 spawned in B and 10 spawned in C. Table III shows the details.

Evidently then the extruded genital products cause the spawning of all ripe individuals in the neighbourhood. In the first and second experiments males

spawned first in the tank containing sperm, so that a spawning male stimulates males as well as females to spawn. In all three experiments males were the first to spawn in the tanks prepared with eggs, so that it is not known whether females

Table II
Experiment commenced at 4.12 p.m.

Times at which urchins spawned	Numbers of urchins which spawned			
	In tank B (containing sperm)		In tank C (containing eggs)	
	Males	Females	Males	Females
4.14	1	—	2	—
4.18	1	—	—	1
4.25	—	1	—	—
4.29	1	—	—	—
4.32	1	—	—	—
4.35	1	—	1	—
4.38	—	—	—	1
4.41	1	—	—	—
4.44	1	—	—	—
4.52	1	1	2	2
4.53	—	—	1	—
5.3	—	—	1	—
5.5	1	1	—	—
5.10	1	—	—	—
5.11	1	—	—	—
5.17	—	3	—	—
5.22	1 which remained without spawning rejected		7 which remained without spawning put into tank C	
6.0	1	1	—	—
6.50	1	2	—	—

Experiment now discontinued. There remained two in tank C which had not spawned.

Table III
Experiment commenced at 5.28 p.m.

Times at which urchins spawned	Numbers of urchins which spawned			
	In tank B (containing sperm)		In tank C (containing eggs)	
	Males	Females	Males	Females
5.32	—	—	2	—
5.46	—	1	2	—
5.51	—	—	—	2
5.56	—	—	2	—
6.0	4	—	—	1
6.5	—	—	—	1
6.15	1	—	—	—
6.22	3	1	—	—
6.50	1	1	—	—

Experiment now discontinued. There remained two in tank B without spawning and four in tank C.

can stimulate other females to extrude their eggs or whether they can excite males only.

This stimulation of males by males is apparently unlike what takes place in heteronereis. Lillie and Just⁽⁴⁾ state that spermatozoa of this annelid present in

the water stimulate females to spawn and that a ripe female which has not yet spawned excites a male to shed its sperm.

Whatever then may be the cause of the simultaneous spawning of echinoids in the sea, whether lunar or non-periodic, it is necessary for the cause to act only on a few individuals, which, by spawning, will excite their ripe neighbours.

It is likely that the secretions from the spermatozoa and eggs which stimulate spawning act through the nervous system of the urchins. There is an aboral nerve pentagon which gives off branch nerves to each gonad. Attempts at direct stimulation of the muscle fibres at the surface of testis or ovary by the application of drops of water containing sperm or crushed eggs failed. Similarly the application of sperm suspension to the genital pores of a number of individuals caused no spawning.

Spawning in one and the same individual takes place at successive full moons in *Centrechinus* at Suez(2), so that ripe genital products are re-formed in four weeks. This is not an abnormally rapid rate of development considering that the Suez water temperature is 26-29°, for Loeb(5) records that in California *Strongylocentrotus purpuratus* re-developes ripe eggs in 10 days. All urchins in a certain area spawned simultaneously and then ripe eggs were unobtainable until 10 days had passed. The water temperature was only 12-15°. I have determined the rate of re-development of ripe eggs subsequent to spawning in *Strongylocentrotus lividus* at Roscoff. On the day following spawning windows were made in the tests of a number of females immediately over an ovary, a small piece of the latter removed for examination, and the windows closed with wax (Koehler's method(3)). The urchins live well after this operation. At intervals of a few days the operation was repeated on the same individuals, a fresh window being cut and closed again on each occasion. In five individuals the first operation, on the day after spawning, showed that the ovaries contained no ripe eggs, while nine days later an operation gave ripe eggs in abundance. The water temperature was 17-19°.

SUMMARY.

(1) The extrusion of the genital products of echinoids is due to the contraction of muscle fibres in the gonad walls, more abundant in the ovary than in the testis. The contraction of these muscles can be artificially stimulated.

(2) A spawning male stimulates ripe individuals of both sexes which are in the neighbourhood to spawn. A spawning female stimulates ripe males to spawn.

(3) After spawning *Strongylocentrotus lividus* re-forms ripe eggs in nine days at a temperature of 17-19°.

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A COMPARISON OF THE MOLECULAR WEIGHTS OF THE PROTEINS

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(Received 6 March 1924.)

(With One Text-figure.)

PROF. WO. OSTWALD has suggested that the calculation of the molecular weights of the proteins can never be attempted with safety. The discrepancies in the calculated results of different authors are certainly large, but the following results show some interesting regularities which require explanation. While working on haemoglobin solutions in the Massachusetts General Hospital, a method was devised on Sörensen's principles which gave accurate results in solutions as dilute as $N/10,000$. The outer liquid was $\cdot 1\text{ N}$ sodium chloride.

Assuming that van't Hoff's law applied to the very dilute solution, the molecular weight was $66,700 \pm 6,000$.

Subject to the limitations given below, it was found that the more concentrated solutions gave the same value for the molecular weight when the following formula was employed:

$$M = [(273 + t) \times 170,000 \times c] \div [(1 - \cdot 03c) \times 273 \times p] \quad \dots(1),$$

t = temperature, c = grams protein per 100 c.c. solution, p = osmotic pressure in mms. mercury. The limits of validity seem to be

$$t = 5 \text{ to } 20, p < 100, pH = 7 \text{ to } 7.5, \text{NaCl} = \cdot 1 \text{ to } 1.0\text{ N} \quad \dots(2).$$

It is possible to give a rational explanation of why the formula should give the true molecular weight within the limits defined above, but for the present, it may be considered merely as an empirical representation of the pressure concentration curve (Fig. 1). Sörensen has pointed out the difficulty of determining the pressure when neither aggregation nor protein salt formation affects the result. It is proposed that the conditions defined above in (2) are an approximate realisation of the environment in which the protein gives its "true" osmotic pressure, provided c is small.

The published molecular weight calculations are based on protein solutions of various concentrations in various mixtures of electrolytes, so the results are not strictly comparable, but the pressure concentration curve for haemoglobin covers a fairly wide range, so that it can be used to effect a partial standardisation.

The following table summarises the results of a number of calculations, in which the experimental material was obtained under conditions resembling those of (2).

Column 1 gives the protein, column 2 the author, column 3 the author's own estimate of the molecular weight, if he has ventured to calculate it. Column 4 gives the molecular weight calculated by the method proposed above. The word "none" indicates that the author has done no experiments which satisfy (2).

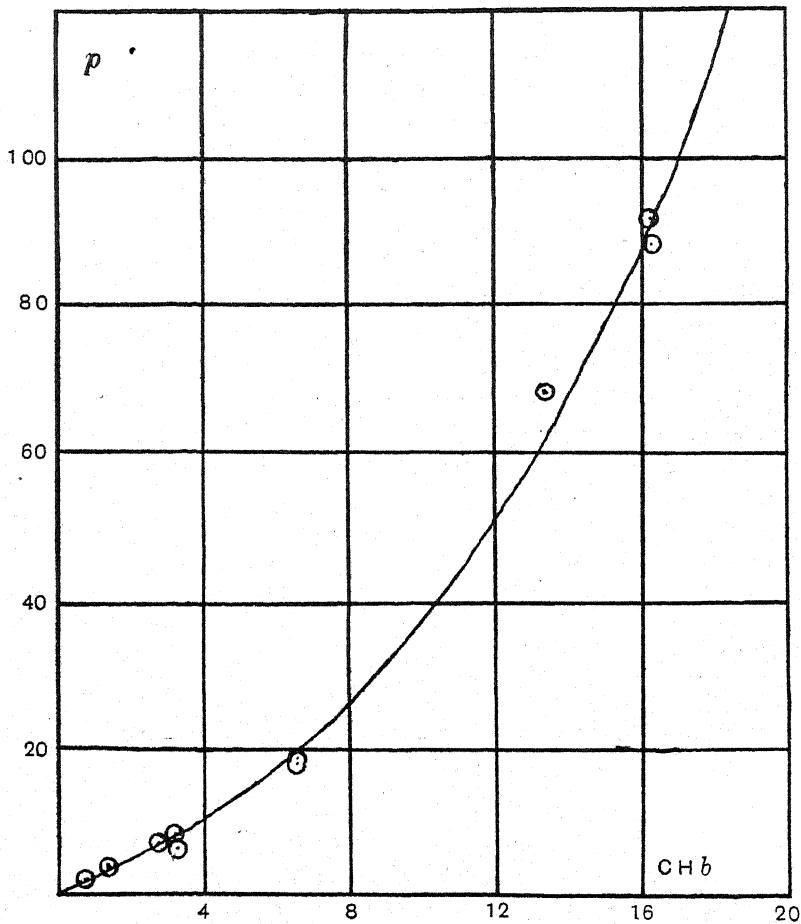


Fig. 1. RELATION OF OSMOTIC PRESSURE AND CONCENTRATION.

Ordinate: Osmotic pressure in mms. of mercury, reduced to 0°C . Abscissa: Concentration in grams Hb per 100 c.c. solution. Horse haemoglobin dissolved in $N/10\text{ NaCl}$. Observed points, circles, line drawn from formula $p = 2.55c/1 - 0.33c$. (Lowest point human Hb.)

In comparing the two columns it will be seen that the results in column 3 are quite irregular, as might be expected with proteins of different composition, while the results in column 4 are all the same within the limits of experimental error.

The only certain conclusion is that more experimental work is required, because the data are scanty and it may be mere chance that such a remarkable resemblance in the molecular weights of various proteins appears in this particular set of experiments.

Some justification should be given for differing from Sörensen in the interpretation of his work on egg albumin. Although the strength of his position is fully appreciated, it will be noted that a very small error in the ammonia nitrogens of Ex. 142 and 144 would vitiate the whole of his argument, and in view of the difficulty of determining small traces of ammonia in the presence of proteins, it seems that Ex. 149 is a better foundation for the calculation of the molecular weight.

Table I

Protein	Author	Mol. wt. as published	Mol. wt. corrected by eqns. (1) and (2)
Egg albumin	Lillie	—	73,000 \pm 15,000
Gelatine	Sörensen	34,000	66,000
"	Lillie	—	68,000 \pm 20,000
Serum protein	Loeb	25,000	None
Oxyhaemoglobin ox	Moore	57,000	80,000 \pm 20,000
Oxy Hb man	Krogh	—	56,000 \pm 15,000
" horse	Hüfner	16,700	None
" sheep	A.	—	66,800 \pm 6,000
Reduced Hb man	"	—	65,000 \pm 6,000
Met. Hb man	"	—	66,700 \pm 6,000
HbO ₂ cow	Roaf	16,000	60,000 \pm 10,000
"	"	99,000	68,000 \pm 6,000
			60,000 \pm 30,000

The fact that the relation of pressure and concentration of haemoglobin is a steep curve may help to account for the differences Krogh records between the serum proteins of different animals, for as the concentrations increase the pressures per 1 per cent. protein may increase also.

The molecular weight estimations of Hüfner and of Loeb are not free from objection.

On the other side, some of the experiments used in column 4 calculation have large experimental errors. Assuming that the experiments planned agree with the theory that the molecular weights of the proteins appear to be in the region of 66,700, it will be necessary to investigate two hypotheses. Firstly, there might be some law of nature which determined the size of protein molecules. Secondly, there might be some peculiarity in protein solutions, which made the osmotic pressure obey the law,

$p = 2.55c$ (when c is small and the solvent $N/10$ NaCl), regardless of the molecular weight of the protein.

SUMMARY.

Applying a new method of calculation, it was found that the molecular weights of ten different proteins were in the neighbourhood of 66,700, a figure very much larger than any previous estimates.

Note. The haemoglobin work was carried out at the Physiological Laboratory, Cambridge, during the tenure of the Reginald John Smith Studentship at King's College, Cambridge, and later at the Massachusetts General Hospital, with the assistance of a Grant from the Procter Fund for the study of chronic disease.

I wish to thank Mr Barcroft and Prof. L. J. Henderson for advice and criticism during this investigation.

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ON THE EARLY DEVELOPMENT OF THE ECHINODERM EGG

I. THE VISIBLE CHANGES FOLLOWING FERTILISATION AND ARTIFICIAL PARTHENOGENESIS ON THE SURFACE OF THE EGG OF *SPHAERECHINUS GRANULARIS*.

By G. S. CARTER, B.A.

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(Received 14 February 1924.)

(With Nine Text-figures.)

INTRODUCTION.

THE observations and experiments which form the subject of this and the two following papers were performed in the Stazione Zoologica at Naples between the months of November, 1922, and March, 1923. In the course of some experiments on *Sphaerechinus granularis* with a different object, some of its eggs were fertilised in sea-water containing a very small quantity of hydrochloric acid. It was observed that no visible change on the surface of the eggs could be seen after fertilisation. Nor did any visible change on the surface occur for some time after the eggs had been returned in natural sea-water; yet the eggs divided in this medium and later produced healthy plutei. Since Loeb (1913 *a*, 1916) had stated his belief that in the echinoderm egg whenever the usual fertilisation-membrane is not formed its place in the activation of the egg is taken by a thin, gelatinous layer which is formed on the outside of the surface and had founded his theory of fertilisation upon this belief, it seemed worth while to investigate more closely in these eggs the relation of the phenomena of fertilisation to the reaction of the sea-water.

A further reason for undertaking this investigation was afforded by the fact that the results of workers at Naples on the problems of fertilisation had frequently differed from those obtained in other places (Loeb, 1913 *a*, p. 222). The results of this investigation agree with those of previous workers at Naples where they come into contact with them, and differ in certain respects from those of American workers.

The present paper, in which the results of the first part of this investigation are described, is concerned with the visible changes which occur on the surface of the egg after development has been initiated. The changes which occur in the presence and absence of a fertilisation-membrane are compared.

It is important to remember that *Sphaerechinus*, the genus on which most of the work described in these papers was done, has been comparatively rarely used in the investigation of the problems of fertilisation. It is particularly suitable for this work since the formation of the fertilisation-membrane appears to be less closely bound up with other changes which occur after fertilisation in its eggs than in those of other genera. Further the limits of the range of conditions in which membrane-formation occurs are particularly sharp in the eggs of this genus.

I should like to take this opportunity of thanking the Managers of the Balfour Fund and Dr G. P. Bidder, for the grant which made possible the carrying out of this work at Naples, and also the Director and Staff of the Stazione Zoologica for every assistance during the course of the investigations.



ZP ---

VM -

CY -

FIG. 1. Surface of the unfertilised egg.

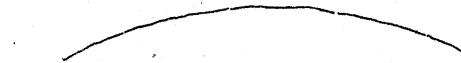
ZP
FM

VM

CY

FIG. 2. Surface of the egg 5 mins. after fertilisation. Development with a fertilisation-membrane.

CH, Chorion. CY, Cytoplasm. FM, Fertilisation membrane. GL, Gelatinous layer. VM, Vitelline membrane. ZP, Zona pellucida.



ZP ---

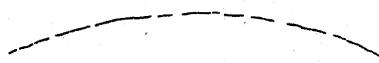
FM ---

VM

CH

CY -

FIG. 3. Surface of the egg 30 mins. after fertilisation. Development with a fertilisation-membrane.



ZP

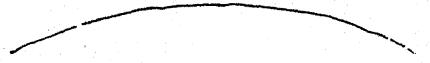
GL

VM

CH

CY

FIG. 4. Surface of the egg 30 mins. after fertilisation. Development without a fertilisation-membrane.



ZP

FM

VM

CY

FIG. 5. Formation of the fertilisation-membrane on the outside of the vitelline membrane.

VISIBLE CHANGES IN THE EGG DURING ACTIVATION WITH AND WITHOUT MEMBRANE-FORMATION.

1. In eggs around which a fertilisation-membrane is formed.

The surface of the unfertilised egg is surrounded by a very thin hyaline layer, the vitelline membrane, and on the outside of this by a thick layer of gelatinous

matter which is very difficult to see in sea-water but becomes visible when Indian ink is added to the sea-water (Gray, 1922). This is the *zona pellucida* (Fig. 1). After fertilisation the fertilisation-membrane appears on the outside of the vitelline membrane and the osmotic layer below it appears to occupy the inner part of the *zona pellucida* (Fig. 2). That the fertilisation membrane arises on the outside

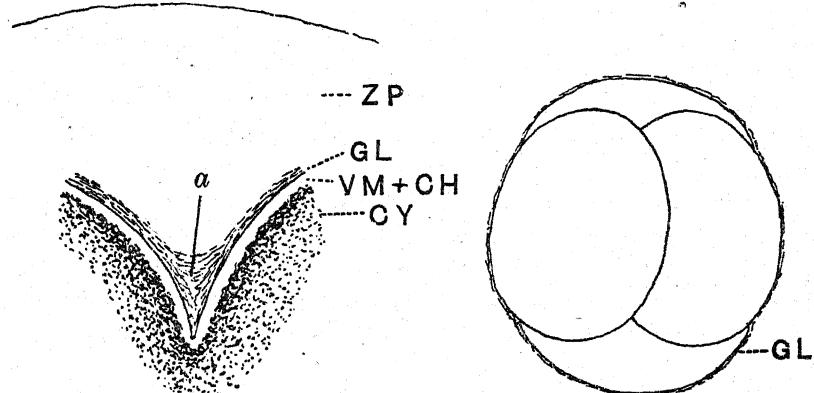


FIG. 6. The first division in development without a fertilisation membrane. Extension of the gelatinous layer between the blastomeres.

FIG. 7. Four-cell stage. Development without a fertilisation-membrane.

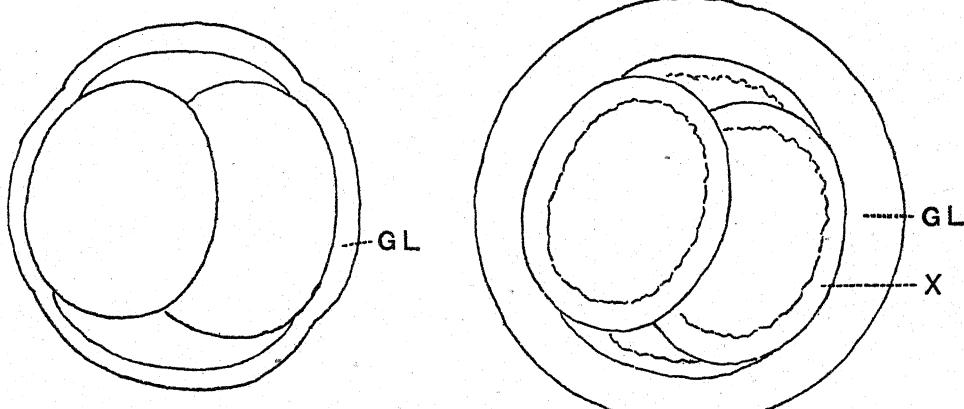


FIG. 8. The same stage as that shown in Fig. 7, treated with chloroform. Gelatinous layer swollen.

FIG. 9. Longer treatment with chloroform. Formation of a membrane held out by osmotic pressure. Blastomeres cytolysing (X).

of the vitelline membrane can be seen most certainly in the eggs of *Sphaerechinus* when it is not formed round the whole circumference of the egg. It then appears to be raised off the outer edge of the vitelline membrane (Fig. 5) which itself remains unaltered and can be recognised much later in the development of the egg. This observation is in disagreement with the statement that has often been made (e.g. Hyman, 1923, for the eggs of *Strongylocentrotus*) that the substance of the vitelline-membrane becomes transformed into the substance to which the de-

velopment of osmotic pressure below the fertilisation-membrane is due. In the eggs of *Sphaerechinus* at least, the larger part of the substance of the vitelline membrane plays no part in the formation of the osmotic layer but it is possible that some substance lying on the outer surface of the vitelline membrane and forming a very thin layer in that position becomes transformed into the substance of the osmotic layer.

About 10-20 minutes after fertilisation a clear hyaline layer collects within the vitelline membrane (Fig. 3), but can be distinguished from it until the egg has divided several times. This is the "chorion." It varies greatly in thickness and is often almost unrecognisable. It is thicker in unhealthy eggs.

In parthenogenetic development the visible surface changes are similar to those described above. The chorion is not normally formed before the egg is placed into hypertonic sea-water. If this is delayed, however, it is formed in the sea-water in which the eggs are placed after treatment with butyric acid but rather later than in fertilisation by the sperm (20-30 minutes after the eggs have been replaced in sea water).

2. *In eggs around which no fertilisation-membrane is formed.*

In these cases there is no visible change on the surface of the egg for about 15-30 minutes after fertilisation or, in parthenogenetic activation, after treatment with the parthenogenetic agent (Fig. 1). At this time a thin gelatinous layer appears on the outside of the vitelline membrane (Fig. 4). This is the "gelatinous layer" which Loeb (1913 *a*, pp. 65, 222) believes to occur in all cases of development in which no fertilisation-membrane is formed and which he believes to represent, in a modified form, the substance of the osmotic layer below the normal membrane. About the same time the chorion appears. At this time the egg is therefore surrounded by a triple layer—the chorion, the vitelline membrane, and the gelatinous layer—and on the outside of these by the zona pellucida (Fig. 4).

The gelatinous layer is visible round these eggs until they have divided at least two or three times. In Fig. 6 it is visible as a thin layer on the outside of the dividing egg and becomes extended in the region between the blastomeres (Fig. 6, *a*).

That this gelatinous layer does in fact represent the material that gives rise to the extrusion of the fertilisation-membrane seems clear from the following observation. Some eggs were fertilised in acid sea-water and allowed to develop until they had reached the four- or eight-cell stage. The gelatinous layer could still be seen on the surface (Fig. 7). They were then treated with sea-water with which chloroform had been shaken up, a medium which caused membrane-formation and cytolysis very rapidly in unfertilised eggs. The gelatinous layer became thicker but for some time kept the outline of the blastomeres (Fig. 8), showing that no membrane held out by osmotic pressure had yet been formed. But in a small proportion of the eggs when this swelling had reached a certain stage, the outside membrane of the gelatinous layer became quite circular and indistinguishable from a normal fertilisation-membrane (Fig. 9). It appeared that some change had occurred in the gelatinous layer and an osmotic pressure had been developed. By

this time cytosis was well advanced in the eggs and they were surrounded by a thick clear layer below the surface (Fig. 9, X). Other parthenogenetic agents such as toluol or benzol, which cause membrane-formation in unfertilised eggs caused the gelatinous layer to swell but did not usually cause the change producing an increase of osmotic pressure within it. The action of these substances in causing membrane-formation is not so violent as that of chloroform.

SUMMARY.

The visible changes at the egg surface during early development with or without a fertilisation-membrane are described. The relation of the substances of the vitelline membrane and of the "gelatinous layer" to the substance to which the extrusion of the fertilisation membrane is due is discussed.

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ON THE EARLY DEVELOPMENT OF THE ECHINODERM EGG

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(Received 14 February 1924.)

II. THE EFFECTS OF CHANGES IN THE SURROUNDING MEDIUM ON THE INITIATION OF DEVELOPMENT AND ON MEMBRANE- FORMATION.

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IN the following paper are contained the results of experimental work upon the eggs of *Sphaerechinus granularis* and other urchins to which the account of the visible changes which succeed the initiation of development given in the preceding paper is preliminary.

PRELIMINARY DISCUSSION.

It has been established as a result of the great quantity of work which has been devoted to the study of the development of the Echinoderm egg that the changes which succeed fertilisation fall into two groups: (1) a series of changes on or near the surface of the egg including those resulting in the sperm-block, the increases of permeability and of consumption of oxygen and the extrusion of the fertilisation-membrane; (2) a group of changes occurring 10-15 minutes after the initiation of development and caused by some action of the sperm within the surface of the egg. In the absence of this second group of changes development does not proceed further than the first or second division.

It is the conclusion of most workers on the subject that the changes of the

first series are not each caused separately by the sperm but are rather members of a cycle which is predetermined in the egg and is allowed to run its course as the result of some change effected by the sperm. This conclusion is supported among other evidence by the very varied nature of the means by which parthenogenesis may be caused. Its truth is generally recognised, but the preliminary change caused by the sperm does not appear to have been given a distinctive name. Since all the changes of the first group take place on or near the surface of the egg, it will be called the *primary surface change* in this paper.

It is with the first group of changes that this paper is concerned.

The earlier part of the paper dealing with the experimental results falls into four parts:

- I. The various effects of alterations in the reaction of the medium on the early development of the egg are described. It is concluded that the absence of a fertilisation-membrane in some circumstances is not a secondary result of any of the other known effects of alterations in the reaction of the medium on the egg.
- II. It is shown that treatment with butyric acid for any period from 10 secs. to 10 mins. will cause the egg to develop but that membrane-formation only results if the period lies within much more narrow limits.
- III. Experiments are described which show that in the presence of KCN membranes may be made to appear in eggs in which development has been initiated without the formation of the membrane up to 30 mins. after the primary surface change has occurred. It is concluded that the substances which normally react to cause the extrusion of the membrane remain unaltered after the primary surface change in the surface of these eggs: and secondly, as a result of this, that the primary surface is quite independent of the change which produces the formation of the membrane.
- IV. Evidence is brought forward that the primary surface change can be caused by treatment which can only be external to the surface of the egg and that it is therefore probably of the nature of a change of the conditions at the surface of the egg and not within it.

In the later part of the paper:

- V. These results are discussed and some suggestions are put forward as to the probable course of events during treatment with the substances which produce parthenogenesis.

The evidence on which these conclusions are based is derived mainly from experiments on artificial parthenogenesis. It seems however clear that the changes in the egg which follow the initiation of development are the same whether caused by the sperm or by artificial means. The conclusion of the fourth section (that the primary surface change is a change of conditions at the surface) is parallel to that of Gray (1922) who suggests that in the case of fertilisation by the sperm the first result of the contact of the sperm with the surface of the egg is an electrical discharge between it and the egg and that it is this discharge which sets in motion the processes leading to the development of the egg.

METHODS.

The urchins were ripe during the whole period of the investigation. Eggs of urchins which had been kept a few days in the aquarium often showed signs of unhealthiness. The animals were therefore collected daily and the experiments performed within a few hours of their capture. The sea-water of the Stazione, which is in continuous circulation and is not taken directly from the sea, was found to give similar results to sea-water collected at some distance from the shore, with which all the results were confirmed.

Precautions were taken in the experiments on artificial parthenogenesis to exclude fertilisation by adventitious sperm. It was found that the water of the circulation in the tap where it entered the aquarium from the storage tanks never contained sperm and this water was always used. Beyond this all the vessels in which the eggs were placed were carefully cleaned and every experiment was controlled by testing the water used for it with unfertilised eggs. The results of this control are recorded in the account of each experiment.

In the standard method of artificial parthenogenesis which Loeb has elaborated, the eggs are treated, after being exposed to the substance which initiates activation (butyric acid), for some time with hypertonic sea-water. In these experiments a hypertonic sea-water consisting of 50 c.c. sea-water + 8 c.c. $5/2\text{ N}$ NaCl was used and the optimum exposure to it for the eggs of *Sphaerechinus* was found to be $2\frac{1}{2}$ hours. That for the eggs of *Strongylocentrotus* and *Echinus* was much less (about 45 minutes). Lyon obtained similar results (1903). Loeb (1900) found that treatment for 2-3 hours with hypertonic sea-water without other treatment caused the eggs of *Arbacia* to develop. It might therefore be suggested that in these experiments the initiation of development was due to the action of the hypertonic sea-water alone. In order to exclude this possibility in each experiment some eggs of the same batch as those used for the rest of the experiment were treated with hypertonic sea-water for the same time as the rest but not with the parthenogenetic agent. If initiation of development due to the hypertonic sea-water occurred, it should have been detected by development among these eggs. In some cases a small percentage of these eggs developed but this percentage was never greater than 2-3 and was therefore not large enough to affect the results. The results of this control are also recorded for each experiment.

It was easy to reduce the period of exposure to hypertonic sea-water by using a more strongly hypertonic sea-water; but when this was done the proportion of the eggs which developed owing to the action of the hypertonic sea-water alone rose in some cases to 10-15 % and for this reason the weak hypertonic sea-water was always used.

The determinations of reaction were made by the Sørensen indicator method using the sulphone-phthaleine indicators proposed by Clark and Lubs (1920). As buffer solutions the phosphate-citric acid mixtures proposed by McIlvaine (1921) were employed over the range $\text{pH } 3.0$ - $\text{pH } 8.0$, and in the more alkaline ranges Sørensen's glycol-NaOH mixtures. The determinations are probably correct to differences of $\text{pH } .2$.

I. INITIATION OF DEVELOPMENT AND MEMBRANE-FORMATION IN RELATION TO THE REACTION OF THE MEDIUM.

1. ACID AND ALKALINE LIMITS OF MEMBRANE-FORMATION.

The formation of the membrane in the egg of *Sphaerechinus granularis* is prevented by a very slight alteration of the reaction of the medium towards the acid side. The limiting value of the reaction varies somewhat with the condition of the urchin and with other factors such as the temperature of the medium, but is always on the alkaline side of neutrality. When fresh urchins were used it was found to vary from $pH\ 7.3$ to $pH\ 8.1$. The reactions of the water of the aquarium and of the sea were both at the time of the experiments $pH\ 8.1$ - $pH\ 8.2$, sometimes almost identical with the limit of membrane formation. Indeed, during cold weather, when the temperature of the laboratory fell considerably below that of the sea, it was often found impossible to obtain membranes in the sea-water itself, whereas the addition of a small quantity of alkali enabled perfectly normal membranes to be formed.

The limit was found to have about the same value when the eggs were activated by the various methods of artificial parthenogenesis. Many of the known methods were used, such as.

- (1) Treatment with a fatty acid, e.g. butyric acid.
- (2) Treatment with the anaesthetics, chloroform, benzol, toluol, or xylol.
- (3) Treatment with such specific chemical parthenogenetic agents as saponin and bile salts.

In every case the limit was extremely precise, normal membranes and complete absence of membranes being often found in sea-waters whose difference in reaction was not greater than $pH\ 2$.

The alkaline limit of membrane-formation was found to be much further removed from the reaction of sea-water. Membranes were formed in sea-water in which the sperm agglutinated in a very few minutes (50 c.c. sea-water + 2.7 c.c. $N/10\ NaOH$). When the eggs were treated with chloroform, membranes were formed in even more alkaline waters (50 c.c. sea-water + 5 c.c. $N/10\ NaOH$ but not in 50 c.c. sea-water + 6 c.c. $N/10\ NaOH$).

In the centre of the range between the acid and alkaline limits of membrane-formation the process is brought about much more easily than at either end of the range. For this reason, membranes are much more certainly formed after treatment with butyric acid if a small quantity of alkali is added to the water to which the eggs are returned.

2. INITIATION OF DEVELOPMENT IN ACID SEA-WATER.

(a) *Fertilisation by the sperm.*

In the study of the phenomena of fertilisation in acid sea-water the difficulty is met that a very slight change of the reaction of the medium towards the acid side inhibits the development of the egg although fertilisation and the primary surface change may have occurred. In the absence of a membrane the changes

which immediately follow the primary surface change are invisible and it is therefore necessary to return the eggs to natural sea-water in order that development may proceed and the fact that fertilisation has occurred become recognisable. If this is done, it becomes necessary to distinguish eggs fertilised in the acid sea-water from those fertilised after the return to natural sea-water by sperm transferred with the eggs. However, eggs which are fertilised in natural sea-water after having been in acid sea-water (less acid than pH 5.2, see below) will form membranes even though fertilised immediately after they are transferred to the natural sea-water. It is therefore possible to distinguish such eggs from those fertilised in acid sea-water, in which membrane-formation does not occur.

When eggs are fertilised in water to which only a very small quantity of acid has been added and are left in this medium, development may sometimes be observed in them. This is due to the "buffer"-action of the dissolved salts which causes the water gradually to regain its original reaction. When this occurs development is able to proceed.

The effects of increasing acidity of the sea-water upon fertilisation are shown by the following experiment. This is an example of four similar experiments.

Experiment 1.

Sperm was added (10.45 a.m.) to some eggs in a series of samples of sea-water to which quantities of hydrochloric acid had been added varying from 0 to 3 c.c. $N/1$ HCl in 50 c.c. sea-water. The eggs were left in these media for one hour. The proportion of the eggs which had formed membranes in that time was estimated (11.45 a.m.) and is recorded in column 3. The eggs were then returned to natural sea-water.

No.	1 Amount of $N/1$ HCl added to 50 c.c. sea- water. c.c.	2 pH	3 Membranes in acid sea-water 11.45 p.m. %	4 Membranes in natural sea- water 12.30 p.m. %	5		7 Membranes in samples 12.45 p.m. %	8 Divisions in samples 4.25 p.m. with membranes %	9 Divisions in samples 4.25 p.m. without membranes %
					Divisions in natural sea-water 2.45 p.m. with membranes %	Divisions in natural sea-water 2.45 p.m. without membranes %			
1	.000	8.1	95	95	99	0	—	—	—
2	.025	7.3	75	95	90	3	—	—	—
3	.250	6.8	0	0	0	75	—	—	—
4	.075	6.2	0	10	10	20	—	—	—
5	.100	5.85	0	75	80	5	—	—	—
6	.125	5.65	0	20	20	20	—	—	—
7	.150	5.5	0	1	3	1	30	30	50
8	.175	5.0	0	0	1	1	80	90	5
9	.200	4.6	0	0	0	1	75	90	0
10	.225	3.9	0	0	0	0	0	0	25*
11	.250	3.5	0	0	0	0	0	0	15*
12	.275	3.3	0	0	0	0	0	0	0*
13	.300	3.1	0	0	0	0	0	0	0*

* In 75 % of these eggs the astral figure was visible.

After a further 45 minutes the proportion of the eggs with membranes was again estimated. In the cases of some of the less acid sea-waters many of the eggs formed membranes after they had been replaced in natural sea-water. These eggs had presumably escaped fertilisation in the acid sea-water owing to the fact that

fertilisation is much slower in these solutions than it is in natural sea-water*. They would then have been fertilised by sperm transferred with the eggs, and would have formed membranes.

Two and a quarter hours later (2.45 p.m.) the proportion of the eggs which had divided with or without membranes was estimated (columns 5 and 6). Those without membranes must have been fertilised in the acid sea-water.

At 12.30 p.m. some eggs which had been in the more acid sea-waters were placed in natural sea-water and more sperm was added to them. Column 7 shows the percentages of these eggs which formed membranes and columns 8 and 9 the percentages which had divided at 4.25 p.m. The results of column 8 show that eggs are unable to form membranes if they have been for one hour in water whose acidity is not less than pH 4.6. Such eggs could still be fertilised.

(b) *The butyric acid method of artificial parthenogenesis.*

Results very similar to those of the last section were obtained in experiments in which eggs were returned to acid sea-water after treatment with butyric acid.

Experiment 2.

The period for which the eggs of a female *Sphaerechinus* should be exposed to the acid solution (50 c.c. sea-water + 3 c.c. $N/10$ butyric acid) to produce the optimum percentage of membranes was determined. Some eggs were then treated with the butyric acid for this period and replaced in a series of acid sea-waters similar to those of the last experiment. After 20 minutes they were placed in hypertonic sea-water (50 c.c. sea-water + 8 c.c. $5/2 N$ NaCl) for $2\frac{1}{2}$ hours and then replaced in natural sea-water.

1 No.	2 Amount of $N/1$ HCl added to 50 c.c. sea-water c.c.	3 pH	4 Membranes in acid sea-water %	5 Divisions		6 Without membranes %
				With membranes %	Without membranes %	
1	.000	8.1	20	10	20	
2	.025	7.3	0	0	60	
3	.050	6.8	0	0	60	
4	.075	6.5	0	0	60	
5	.100	6.1	0	0	50	
6	.125	5.6	0	0	50	
7	.150	5.0	0	0	75	
8	.175	4.6	0	0	0	7
Control 1. Eggs treated with hypertonic s.w. and not butyric acid						
Control 2. Eggs placed in the natural s.w. used in the experiment. (Control for presence of advegilous sperm.)						

Column 4 gives the percentages of eggs which formed membranes in the acid sea-water and columns 5 and 6 the percentages which had divided in the three hours succeeding the treatment with the hypertonic sea-water.

* Clowes and Smith (1922) find that the permeability of the sea-urchin egg to sperm of the same species varies with the pH of the medium.

This experiment is an example of seven similar experiments.

A similar result was obtained with *Strongylocentrotus*.

On this experiment the following notes may be made.

(1) In all experiments dealing with artificial parthenogenesis irregular divisions were very frequent. *Sphaerechinus* is not an urchin in which it is easy to initiate quite healthy development parthenogenetically. It was clear that the irregularity of the divisions was due to the fact that the appropriate time for which the eggs must remain in hypertonic sea-water is not constant for different females and that the margin of error is not great. Regular development could be obtained if the treatment with hypertonic sea-water was of exactly the right length.

(2) Loeb (1913 a) found that eggs of *Arbacia*, which had been treated with butyric acid and placed in acid sea-water forming no membranes, returned to the unfertilised condition after some time in the acid sea-water and could later be fertilised. This is perhaps parallel to the observations of American authors (Just, 1921) that membranes may be formed by fertilisation with sperm in eggs which have been treated with butyric acid for too long a time to allow membrane-formation on return to sea-water. In neither of these cases are membranes usually formed in the eggs of *Sphaerechinus*. When sperm is added to the eggs which have been returned to acid sea-water after treatment with butyric acid, a small proportion sometimes form membranes but the great majority are quite unfertilisable. It is not clear that this small proportion has not merely escaped the activation due to the butyric acid. Lillie (1917) obtained similar results with the eggs of asteroids.

3. DISCUSSION.

(a) Range of reaction in which the primary surface change can occur.

That fertilisation may occur in Echinoderm eggs in sea-water of which the reaction is too acid to allow the formation of the fertilisation-membrane has often been observed. In the case of *Sphaerechinus* it was observed by Herbst (1906) that eggs could be fertilised without membrane formation in slightly acid sea-water. The experiments described above show that the primary surface change can be caused by fertilisation in water of any acidity up to $pH\ 5.65$ or parthenogenetically in water at least as acid as $pH\ 5.0$. Thus:

(1) *Fertilisation.* It was shown above that the eggs in experiment 1 which developed without membrane formation (column 6) must have been fertilised in the acid sea-water. Fertilisation can therefore occur in water of $pH\ 5.65$ (column 6, No. 6).

(2) *Parthenogenesis.* It has been suggested that the primary surface change takes place immediately after the egg is placed *into* the solution of the parthenogenetic agent. If this is accepted it is necessary to conclude that this change occurs in water of the acidity of this solution ($pH\ 4.4$ - $pH\ 4.6$ for the case of butyric acid). If it is not accepted, the results of experiment 2 show that it can occur in water of any acidity up to $pH\ 5.0$: for large proportions of the eggs returned to waters of these acidities were able to develop when later placed into natural sea-water. It seems clear that this development was due to the butyric

acid and not to the acid sea-water to which the eggs were returned. For although it is shown below that treatment with acid sea-water alone is able to cause the initiation of development, it is also shown that the least acid sea-water which is able to do this is of $pH\ 5.2$. The eggs of at least the first six batches in experiment 2 could not have been activated by this means.

(b) *The various effects of abnormal acidity of the medium upon the sperm and the egg.*

The known reactions of the egg and the sperm to abnormal acidity of the medium which might cause the absence of a visible membrane are as follows*:

(1) The activity of the sperm is reduced in acid sea-water and, if the acidity is sufficiently great, fertilisation is prevented thereby.

(2) The radius of the fertilisation-membrane (*i.e.* the thickness of the osmotic layer) is reduced in acid sea-water (Gray, 1922).

(3) The mechanism of membrane-formation is destroyed when the acidity of the sea-water exceeds a certain limit. Eggs which have been treated with water more acid than this limit and returned to natural sea-water are fertilisable but will form no membranes.

The different values of the reaction of the medium which produce these three changes are determined by the results of experiment 1 and by some further results recorded below.

(1) The results of column 6 (experiment 1) show that fertilisation was prevented in sea-water more acid than $pH\ 5.65$. Since eggs of these batches could develop when treated with fresh sperm (column 7), it follows that the egg was undamaged by the treatment and fertilisation was prevented by some action on the sperm or on its power of causing activation in the egg. That the primary surface change can occur in waters of these acidities has been shown in the experiment with butyric acid (Exp. 2), it must be concluded therefore that fertilisation was prevented in these eggs by some action on the sperm and presumably by the reduction of its activity.

(2) The reduction of the thickness of the osmotic layer is much less in *Sphaerechinus* than that which has been observed by Gray (1922) in *Echinus*. He found it reduced to 16% of its value in natural sea-water when the reaction is brought to $pH\ 6.9$. In *Sphaerechinus* it is reduced to about 80–85% of its normal value in water of this acidity and in more acid waters there is no further reduction. This is true until a reaction of $pH\ 3.3$ is reached beyond which the investigation was not carried. In water of this reaction the egg quickly shows abnormal changes.

(3) Eggs which had been in water of $pH\ 4.6$ for one hour could still form membranes (Exp. 1, column 7, No. 9): in more acid sea-waters the mechanism of membrane-formation was destroyed. Further experiments showed that the least acid sea-water which was able to cause this change was of $pH\ 5.0$ – $pH\ 5.2$. In water of this acidity the change occurs extremely slowly (after 2–3 hours) but,

* I am indebted for this classification of the effects of acidity of the water upon fertilisation to Mr J. Gray, M.A., of King's College, Cambridge, who has kindly allowed me to use some unpublished results for the purposes of this paper.

as the acidity is raised above this point, the speed of the change increases rapidly. As in most other respects females differ somewhat in the exact values of the reactions at which these changes occur in their eggs.

(c) *The cause of the absence of a visible membrane in eggs fertilised in acid sea-water.*

From the above discussion it is clear that the absence of a visible membrane in these eggs is not due to the destruction of the mechanism of membrane-formation in acid sea-water (this does not occur in water less acid than pH 5.2), nor is it due to the reduction of the thickness of the osmotic layer which is never complete in the eggs of *Sphaerechinus*, nor to inability of the sperm to fertilise the eggs.

This phenomenon is therefore due to some effect of the sea-water on the egg which is not included in the above list. Since it is an immediate result (eggs fertilised one minute after they are placed in the acid sea-water will produce no membranes) and it may be caused by the addition of any acid, mineral or organic, it seems that it is due to some alteration of the surface conditions of the egg whereby the reaction which leads to the extrusion of the membrane is prevented.

II. THE PRIMARY SURFACE CHANGE IN THE BUTYRIC ACID METHOD OF ARTIFICIAL PARTHENOGENESIS.

It is shown by the following experiment that butyric acid causes the primary surface change in an exposure of 10-20 seconds but that membrane-formation does not result unless the exposure is somewhat longer.

Experiment 3.

Some eggs of *Sphaerechinus* were treated with butyric acid (50 c.c. sea-water + 3 c.c. N/10 butyric acid) for times varying from 10 seconds to 10 minutes. The eggs were then replaced into sea-water for 15 minutes and thence placed into hypertonic sea-water (50 c.c. sea-water + 8 c.c. 5/2 N NaCl) for 2½ hours. They were

No.	Period in butyric acid	Membranes formed in sea-water %	Divisions	
			With membranes %	Without membranes %
1	10 secs.	0	0	12
2	20 "	0	0	50
3	30 "	60	50	15
4	40 "	30	25	30
5	50 "	10	5	5
6	1 min.	30	25	30
7	1½ mins.	0	0	40
8	2	0	0	50
9	2½ "	0	0	35
10	3	0	0	60
11	3½ "	0	0	40
12	4	0	0	15
13	6	0	0	50
14	8	0	0	70
15	10 "	0	0	35
16	Control 1. Eggs treated with hypertonic sea-water and not butyric acid	0	0	0
17	Control 2. Eggs placed in the natural sea-water used in the experiment	0	0	0

then again replaced into sea-water and four hours later the percentage which had divided with or without membranes was estimated.

This experiment is an example of five similar experiments.

A similar result was obtained with *Strongylocentrotus*.

III. THE RELATION OF MEMBRANE-FORMATION TO THE PRIMARY SURFACE CHANGE.

I. DISCUSSION.

From the results of the preceding sections it is clear that the conditions under which a normal fertilisation-membrane is formed in these eggs are somewhat limited in comparison with those in which the initiation of development takes place.

Loeb (1913 a) in discussing these cases suggested that the formation of the gelatinous layer takes the place of normal membrane-formation and that either one or other of these two processes always occurs and is an integral part of the initiation of development. But Shearer (1922) has shown that the increased consumption of oxygen, which is undoubtedly a member of the series of changes which take place in the egg after fertilisation and which must therefore follow the first member of that series, the primary surface change, can be observed within the first minute after the sperm is added to the eggs. In *Sphaerechinus* no trace of the gelatinous layer is visible on the surface of the egg within the first 15 minutes after the development has been initiated. A similar observation was made by Gray in *Echinus* (unpublished results). It seems then that at least the visible appearance of this layer can play no part in the primary surface change, but belongs rather to the second series of changes which occur in the egg 10-20 minutes after fertilisation and are most obviously characterised by the formation of the "chorion."

This reasoning does not exclude the possibility that there is concerned in the primary surface change some previous invisible reaction between the substances which normally react to give rise to the extrusion of the membrane, but there seems no evidence for such a view. It seems more probable that the view put forward by Lillie (1919, etc.) is correct, namely that the primary surface change is quite independent of the formation of the membrane.

In any case it is clear that membrane-formation is dependent upon the *previous occurrence* of the primary surface change, for membranes are never formed in unfertilised eggs. But this dependence might be due to some alteration of the conditions at the surface of the egg which results from the primary surface change and is necessary for the success of the reaction which gives rise to the extrusion of the membrane. If this were so, it would not be unnatural that in some conditions the primary surface change might occur and the surface still remain unsuitable for the extrusion of a membrane. In such conditions the mechanism which normally gives rise to the formation of a membrane having failed to perform its function might remain undamaged in the surface of the egg and might be made to perform its function at a later stage. If this were so, it would seem clear that the primary surface change is quite independent of the reaction which results in the extrusion of the membrane.

The observations of several authors (e.g. Just, 1921, on *Arbacia*) that membranes may be formed in eggs which have previously started to develop by later fertilisation with sperm seem to support this view.

Attempts to obtain similar results with the eggs of *Sphaerechinus* at Naples failed. Except in a small percentage of eggs which had apparently escaped activation by the butyric acid, no membranes were formed whether the eggs were treated with parthenogenetic agents such as chloroform, benzol, saponin, etc., or sperm was added. This was found to be the case when the eggs had been treated with butyric acid only three or four minutes previously. When however the same experiments were repeated on the eggs of *Echinus miliaris* at Plymouth in August, 1923, many membranes were formed.

It therefore seems to be the case that in some genera the reaction which gives rise to membrane-formation can occur some time after the primary surface change and that the two changes are therefore distinct. In *Sphaerechinus granularis* membranes can be formed only immediately after the primary surface change and, since if the changes are distinct in the one genus they are probably distinct in the other, it seems probable that in *Sphaerechinus granularis* this failure to form the membrane is due to some further change which occurs in the egg shortly after the primary surface change and that there is for this reason only a short period after the primary surface change during which membrane-formation can occur.

Now during this short period after the primary surface change, the best-known changes in the egg are the increases of permeability and of oxygen consumption. It therefore seemed possible that one of these changes was responsible for the hypothetical alteration of conditions preventing the formation of the membrane. The increase of permeability is largely prevented if the eggs are placed into slightly acid sea-water (Gray, 1916) but no membranes are formed if activated eggs are treated with chloroform in such a solution. It does not therefore seem probable that this change is the cause of the inhibition of membrane-formation. There remains the increase of oxygen consumption.

2. EXPERIMENTS WITH SEA-WATER CONTAINING KCN.

Loeb (1906) showed that the consumption of oxygen in the fertilised egg is greatly reduced when the egg is placed in sea-water in which KCN is present. The consumption of oxygen in these circumstances falls to not more than 40 % of its value in normal conditions. That the increase of permeability is similarly reduced by treatment with KCN is denied in *Arbacia* by Loeb and Warburg (Loeb, 1913 a). Experiments were therefore performed to discover whether membrane-formation was possible in eggs activated without it when they were placed in a dilute solution of KCN.

The following results show that this is so.

Experiment 4.

Some eggs were placed in a solution containing butyric acid (50 c.c. sea-water + 3 c.c. N/10 butyric acid) for four minutes. This period was too long to allow membrane-formation, and no membranes were formed when the eggs were

replaced in sea-water. Some of the eggs were placed after 1, 3, 5, 7, 9, 35 and 55 minutes in sea-water into two solutions:

(1) Sea-water shaken up with toluol, a solution which caused normal membrane formation in untreated eggs.

(2) 50 c.c. sea-water + 1 c.c. 1/10 % KCN. This strength of KCN solution was chosen as that found by Loeb effective in reducing the oxygen consumption in fertilised eggs.

After ten minutes in the last solution the eggs were transferred to:

(3) A solution similar to (2) with which toluol had been shaken up and which also caused membrane-formation in untreated eggs. The reaction of this solution was unaltered by the addition of toluol and the formation of membranes in it therefore could not be due to changes in the reaction.

The table shows the percentage of membranes that were formed in each solution.

This experiment is an example of five similar experiments.

Period for which the eggs remained in sea-water (mins.)	Membranes formed in the solutions		
	(1) Sea-water + toluol %	(2) KCN sea- water %	(3) KCN sea-water + toluol %
1	20	50	90
3	5	0	55
5	3	0	50
7	2	0	45
9	5	0	40
33	12	1	40
55	10	0	10

Some of the original eggs ten minutes after they had been replaced in sea-water were placed in hypertonic sea-water, left in this for 2½ hours and then replaced in natural sea-water. 70 % of these eggs divided (without membranes) showing that at least this proportion had undergone activation in the butyric acid solution. Controls showed that no eggs had been activated by the hypertonic sea-water alone or by adventitious sperm.

Later sperm was added to some of the original eggs and 10 % formed membranes, having apparently escaped activation in the butyric acid solution. This proportion agrees approximately with that of the eggs which formed membranes in the sea-water containing toluol but not KCN (column 1). Unactivated eggs had been shown to form membranes in this solution and these eggs were therefore presumably unactivated.

In the KCN solution practically no membranes were formed except in the case of eggs which had been only one minute in sea-water. But in this case the butyric acid introduced within the surface of the egg would probably not have had time to diffuse away. In the light of the results given in the third column which show that toluol in the presence of KCN caused membrane-formation, it seems probable that the membranes in this case were due to the presence of this butyric acid*.

* It is possible that the slightly greater alkalinity of the KCN sea-water as compared with natural sea-water (a difference of $pH\cdot2$) might be responsible for membrane-formation in these eggs.

The percentage in which membranes were formed by toluol in the presence of KCN became smaller the longer the time the eggs were left in sea-water after the treatment with butyric acid, presumably due to later changes in the egg. The larger fall in this proportion after 30 minutes in sea-water may be associated with the formation of the gelatinous layer about this time.

It is usually assumed that, when eggs are treated for too long a time with butyric acid, the reason that no membranes are formed when the eggs are returned to sea-water is that either the egg or the mechanism of membrane formation within it has been damaged by too great a penetration of the acid. But this experiment has shown that merely placing such eggs in sea-water containing KCN will allow membranes to be formed if butyric acid is still present in the egg (column 2, No. 1) and this would not be possible if the mechanism had been destroyed. There is another possibility. It is suggested below that initiation of development is due in the first instance to a change of the surface-conditions of the egg brought about by the action of the H^+ and (butyric) $^-$ ions when the eggs are put *into* the acid solution. Further it is suggested that this change is followed in the acid solution by the changes which underlie the increase in permeability and in oxygen consumption though at least the former of these does not occur in the acid solution. If one or both of these underlying changes prevent the formation of the membrane when they have gone on for more than three or four minutes (and we have seen that the failure to form a membrane is reversed when the increase of oxygen consumption is reduced), the fact that no membrane-formation is possible after the eggs have been more than three or four minutes in the butyric acid solution would be accounted for.

The observation that a higher percentage of the eggs form membranes in the toluol solution after only one minute in sea-water (column 1, No. 1) was confirmed by further experiments. It could be explained on the above theory either on the ground that:

(1) Toluol has a more violent action on the egg than has butyric acid (and this is clear from the rapid cytolysis it causes). If this is so, it might be expected to cause membrane-formation for a longer period after the initiation of development. Or (2) Toluol and butyric acid if present in the surface at the same time have a similar strong action.

Similar results to those recorded in Exp. 1 were obtained when initiation of development was caused without membrane-formation by treating the eggs for too short instead of too long a time with butyric acid or when the eggs of *Strongylocentrotus lividus* or *Echinus microtuberculatus* were used. It was not possible to obtain conclusive results from similar experiments on eggs fertilised by sperm in acid sea-water for the reasons that (1) fertilisation is very slow in these solutions and only a small percentage of the eggs at any one time have been fertilised and have not yet developed the gelatinous layer and (2) it is impossible to distinguish fertilised from unfertilised eggs until the gelatinous layer is formed.

The experiment in which the eggs were treated for too short a time with butyric acid is of interest since it confirms the conclusion drawn from the experi-

ments described below that initiation of development is caused by an exposure to the acid which is too short to cause the formation of a membrane. This subject is further discussed in Section III.

Experiment 5.

Similar to Exp. 4. *Strongylocentrotus*. Exposure to the water containing butyric acid one minute. 2 % of the eggs formed membranes in sea-water.

Period for which eggs remained in natural sea-water (mins.)	Membranes formed in solutions		
	(1) Sea-water + toluol %	(2) KCN sea- water %	(3) KCN sea-water + toluol %
1	70	2	90
3	1	2	10
5	2	2	65
7	2	2	50
30	5	2	35

It is noteworthy that in this experiment no larger percentage of membranes are formed in the eggs put into KCN sea-water after one minute than in those which remained longer in the sea-water. This was to be expected, for the butyric acid not having penetrated sufficiently to cause membrane-formation in the natural sea-water would not do so in the KCN sea-water.

If the presence of KCN allows the membranes to be formed for a longer period after the primary surface change than is otherwise the case, it should follow that eggs treated with butyric acid for too long a period to allow membrane-formation in sea-water, should form membranes if KCN is present in the water to which they are returned. Some experiments on these lines were performed on the eggs of *Sphaerechinus* with the expected results, but the fact was overlooked that KCN increases the alkalinity of the sea-water to which it is added. Since increase in the alkalinity of the sea-water also prolongs the period for which the eggs may remain in the acid and still form membranes, the results were inconclusive.

These experiments were repeated at Plymouth in August, 1923, with the eggs of *Echinus miliaris* and gave the following results. As an example of three similar experiments the following is given.

Experiment 6.

10 c.c. 1/10% KCN were added to 500 c.c. sea-water and the reaction of this water was found to be pH 8.7. Some sea-water was then brought to the same alkalinity by the addition of N/10 NaOH. Eggs were then treated with butyric acid for different periods and placed in these two media. The percentage of membranes in each case is recorded in the following table.

It is clear from this table that the eggs will form membrane after a longer

Period in butyric acid	secs.	5	10	15	20	30	45	min.	1	1½
Sea-water without KCN	%	0	0	0	75	75	80	85	85	80
Sea-water with KCN	%	0	0	0	10	65	60	80	80	80
Period in butyric acid	mins.	2	3	4	6	8	11	14	17	20
Sea-water without KCN	%	70	40	25	3	7	0	4	4	5
Sea-water with KCN	%	90	65	75	2	0	60	75	50	80
Period in butyric acid	mins.	23		26	30	35	40	50	60	
Sea-water without KCN	%	0		0	0	0	0	0	0	
Sea-water with KCN	%	70		35	3	0	0	0	0	

period in the acid solution if KCN is present in the water to which they are returned.

The membranes formed in the KCN sea-water around eggs which had been 20-30 minutes in the butyric acid were unusually close though quite definitely present.

3. CONCLUSIONS.

The last experiment shows that the presence of KCN is favourable to the formation of the membrane even in those cases in which the membrane may be formed for some time after the primary surface change. It may therefore be supposed that the presence of KCN is in general favourable to the formation of the membrane in the developing egg. On account of the well-known action of KCN in reducing oxidations in the egg and in other living matter, it seems probable that this effect is indirect and due to a reduction of the oxidations, but the possibility is not excluded that it is due to some direct action of the substance itself.

The experiments described in this section show:

(1) That in eggs, in which no membranes are formed immediately after the primary surface change, membranes can be produced by treatment with a parthenogenetic agent in the presence of KCN within the first 30 minutes after the primary surface change, and therefore:

(2) That the mechanism of membrane-formation is undamaged after the primary surface change has occurred in these eggs.

From these results it is concluded:

(1) That membrane-formation is only secondarily associated with the primary surface change.

(2) That changes occur in the developing egg of *Sphaerechinus* and to a less extent in the eggs of other urchins, three or four minutes after the primary surface change which are unfavourable to membrane-formation.

(3) That these changes are probably associated with the increased consumption of oxygen in the egg, since they are reversed together with it by the presence of KCN in the sea-water surrounding the egg.

IV. THE NATURE OF THE PRIMARY SURFACE CHANGE.

I. DISCUSSION.

From Exp. 3 it is clear that treatment with butyric acid for any period from 10 secs. to 10 mins. will cause activation but that membrane-formation is caused only when the period lies within much more narrow limits.

The rôle of the acid must therefore be a double one—firstly to cause almost instantaneously the change which initiates development and secondly to cause the reaction which results in the extrusion of the membrane.

That the butyric acid causes at least two separate changes in the egg is supported by the evidence brought forward by Just (1920) who found that in the case of *Echinorachnius* eggs treated for too short a time with butyric acid are altered by the treatment in that they are more resistant to later cytolytical changes than are untreated eggs. He concludes:

"The effect of the butyric acid is not a reversible destructive reaction in need of a 'corrective factor' but a constructive, irreversible, practically instantaneous reaction setting in motion the whole train of events with accompanying increases, in oxidation, permeability, etc. leading to the cleavage of the egg."

Loeb has shown that the parthenogenetic agent causes membrane-formation by penetration within the surface of the egg and action upon some substance below it. Since the primary surface change is distinct from membrane-formation, the question is still open whether it is caused in the same way or by some action of the parthenogenetic agent on the outside of the surface causing a change of the conditions of the surface itself.

It has often been suggested that the changes induced in the egg by the sperm are of the nature of surface changes of this kind (R. S. Lillie, McClendon, etc.). Such suggestions, apart from Loeb's conclusive evidence that the extrusion of the fertilisation-membrane in artificial parthenogenesis is the result of penetration of the parthenogenetic agent below the surface of the egg, have been met by F. R. Lillie's proof that a chemical substance, "fertilizin," is essential to the initiation of development by the sperm. But Lillie's evidence does not seem to exclude the possibility that the first change which occurs after fertilisation is a change of the surface conditions and that the change which he calls the "activation" of the fertilizin follows either directly as a result of this change, as would seem probable in the cases of parthenogenesis to be described immediately, or possibly as a result of some further action of the sperm or parthenogenetic agent.

There is a certain amount of evidence that treatment which can hardly be penetrative may initiate development parthenogenetically. For instance, Loeb in some of his earliest experiments (1900) found that he could cause a few divisions without membrane-formation in the eggs of *Arbacia* by treatment for 10 minutes with sea-water acidulated with hydrochloric acid. Again Lyon (1903) working at Naples found that in *Arbacia* and *Sphaerechinus* hydrochloric acid was an effective agent in causing activation. He obtained development in 10 % of the eggs of *Arbacia* by exposure for periods varying from 5-15 minutes to HCl solutions of

strengths varying from $7/1000$ N. to $2/1000$ N. With *Sphaerechinus* he obtained "much segmentation and a few good plutei" by similar treatment. No mention of fertilisation-membranes is made in his paper but Loeb (1913, p. 222) states he believes "gelatinous membranes" were formed.

2. EXPERIMENTS WITH SEA-WATER ACIDIFIED WITH HCl.

In view of the facts stated above it seemed worth while to compare the effects upon the egg of *Sphaerechinus* of treatment with sea-water to which hydrochloric acid had been added with the results obtained with butyric acid.

The result of many such experiments was that the initiation of development was caused by treatment with hydrochloric acid but in a smaller proportion of the eggs than in the case of butyric acid.

The proportion varied from 0 to 30 or 40 % of the eggs of different females; the average value was about 10-15 %. Further experiments showed that development was initiated whether the eggs were replaced after treatment with the acid into natural sea-water or into a series of acid sea-waters, and again that the acid was effective after an exposure in some cases of as little as 10 seconds and usually in 20 seconds. In only a few occasional cases were membranes formed.

The following are typical results.

Experiment 7.

Some eggs were placed in 50 c.c. sea-water + 2 c.c. N/1 HCl (pH 4.2)* for periods varying from 10 secs. to 10 mins. They were then replaced in sea-water for 20 mins. and thence put into hypertonic sea-water (50 c.c. sea-water + 8 c.c. $5/2$ N NaCl) for $2\frac{1}{2}$ hours, after which they were replaced into natural sea-water. After a further four hours the percentage of eggs which had divided was estimated. In this case small percentages of membranes were formed in some of the tubes. The proportion of eggs which developed was above the average.

This is an example of six similar experiments.

No.	Period in HCl sea-water	Membranes %	Divisions %
1	19 secs.	0	15
2	20 "	0	15
3	30 "	0	20
4	45 "	0	10
5	1 min.	0	10
6	1 $\frac{1}{2}$ mins.	0	30
7	2 "	0	20
8	2 $\frac{1}{2}$ "	0	15
9	3 "	10	20
10	3 $\frac{1}{2}$ "	5	35
11	4 "	5	30
12	6 "	3	30
13	8 "	0	35
14	10 "	0	50
15	Control 1. Eggs treated with hyper-tonic sea-water and not butyric acid	0	1
16	Control 2. Eggs placed in the natural sea-water used in the experiment	0	2

* An acid solution of this strength was used as having the same acidity as the butyric acid solution usually employed (pH 4.2-pH 4.4).

Experiment 8.

Some eggs were placed in 50 c.c. sea-water + 175 c.c. N/1 HCl (pH 4.5) for two minutes and thence into sea-water made acid with varying quantities of HCl for 15 mins. They were treated with hypertonic sea-water for $2\frac{1}{2}$ hours and replaced into natural sea-water. No membranes were formed. The percentage of divisions was estimated after $2\frac{1}{2}$ hours in this last medium.

Five similar experiments were performed.

No.	Strength of HCl in acid sea-waters	pH	Divisions %
1	.000	8.3	3
2	.025	7.0	15
3	.050	6.6	3
4	.075	6.4	10
5	.100	6.1	20
6	.125	5.8	3
7	.150	5.0	10
8	.175	4.5	12
9	.200	4.1	5
10	Control 1. Eggs treated with hypertonic sea-water and not butyric acid	—	—
11	Control 2. Eggs placed in the natural sea-water used in the experiment	—	0

This result is irregular but shows that the initiation of development is possible when the eggs are returned after treatment with the acid sea-water to waters of any acidity up to that of the acid sea-water itself. It must be remembered that the percentage of divisions gives only a minimum for the percentage of the eggs in which the primary surface change had occurred and not an exact estimate of this percentage. Probably in many of these cases the low percentages of divided eggs were due to some failure of the later treatment rather than to failure of the acid sea-water to cause development.

Further experiments showed that the least acid sea-water which caused the initiation of development had a pH of 5.0 or 5.2. In cases 1-6 of this table, therefore, the initiation of development could not have been due to the second solution.

3. CONCLUSIONS.

(a) These results are parallel in two respects to those obtained with butyric acid and described above:

- (1) The initiation of development results after a treatment of only 10 secs. with the acid.
- (2) The initiation of development is not prevented by abnormal acidity of the sea-water into which the eggs are placed after treatment with the acid. This is in contrast with the inhibition of membrane-formation in acid sea-water.

(b) These experiments show that the initiation of development can be caused by treatment which can only be external to the surface of the egg. It seems necessary to conclude that in these cases the primary surface change must be a change of the conditions at the surface of the egg and not within it.

V. GENERAL DISCUSSION.

The solution of butyric acid used in the standard method of artificial parthenogenesis contains H^+ ions in approximately the same concentration as the acid sea-water used in the experiments of Section IV.

The primary surface change must be of the same nature in the two cases. It therefore seems probable, since the initiation of development can be caused by the H^+ ions alone, that in the case of treatment with butyric acid the H^+ ions play some part in causing the change. There is the difference between the two cases that the treatment with butyric acid causes initiation of development in a much larger percentage of the eggs than does hydrochloric acid. This further effect is presumably due to some action of the butyric ions.

Another question is raised by these experiments. It was found that the initiation of development followed the treatment with acid sea-water when the eggs were replaced into sea-water of any reaction from pH 5.4 to pH 8.1. The only change to which the eggs are subjected in being taken out of the first solution and placed into the second is the change in reaction (since the great mass of Cl^- ions present prevents the change in their concentration being appreciable). The initiation of development in the eggs bears no relation to the size of this change (it was found to occur when the reaction of the first solution was pH 5.2 and of the second pH 5.4) and for this reason it seems improbable that it is caused by it. The only other change to which the eggs are subjected during the treatment is the initial increase in acidity when they are placed into the first solution. It becomes necessary to enquire whether it is possible that it is this change which causes the primary surface change in the egg.

It is usually assumed that activation does not occur until the eggs are taken out of the acid solution because none of the changes which follow activation are recognisable in the acid solution. But it can be shown on the evidence at hand that each of these changes except perhaps the increase of oxygen-consumption would be separately inhibited by the acidity of the solution and would therefore be unrecognisable in it.

The immediate results of the primary surface change are the sperm-block, the increases of permeability and oxygen-consumption and the extrusion of the membrane.

(1) The sperm-block does not enter the question in artificial parthenogenesis since no sperm is present.

(2) That the increase of permeability does not occur in the acid solution is a necessary result of Gray's observation (1916) that this change is always greatly and reversibly reduced by abnormal acidity of the sea-water.

(3) The consumption of oxygen in the fertilised egg of *Strongylocentrotus* was shown by Warburg (1910) to vary with the reaction of the medium. The values he obtained were 1.4 at pH 6.0, 3.9 at pH 8.0 and 8.1 at pH 11.0. It may be said therefore that the oxygen consumption of the developing egg is reduced in the acid solution. This reduction is very much less than the increase found by Shearer (1922) for the consumption of oxygen in the fertilised as compared with

the unfertilised egg (8000 %) and it is possible that an increased oxygen consumption might be found in the eggs while they are in the butyric acid solution.

(4) It is shown above that membrane formation is inhibited in sea-water more acid than pH 7.3.

If then the primary surface change occurred when the eggs were put into the acid solution none of its results except perhaps the increase in oxygen consumption would be recognisable until the eggs were replaced into sea-water. Since the change is itself invisible, the fact that there is no recognisable change in the eggs in the acid solution is no evidence that it has not occurred.

That in the acid sea-water a change takes place in the egg which prevents the formation of the membrane in the standard method of parthenogenesis after it has gone on for more than 3-4 minutes, was the conclusion drawn from the results of the experiments with KCN (p. 13). This change is reversed by the action of KCN and is therefore probably associated with the increase of oxygen consumption in the egg. If this is so the changes which underlie the increase in oxygen consumption must occur in the acid sea-water. This would support the contention that the primary surface change has occurred while the eggs are in the acid solution.

The argument of this section leads to the following suggestions as to the course of events in the eggs during the treatment with butyric acid.

(1) When the eggs are placed into the acid solution a change of the surface conditions (the primary surface change) immediately occurs, caused in part by the H^+ ions, in part by the (butyric)⁻ ions.

(2) As a result of this change conditions of the surface are left which allow the increase of permeability to occur when the eggs are replaced into natural sea-water, but this increase is inhibited in the acid.

(3) Changes go on within the surface which underlie the increase of the oxidative processes and lead to the increased consumption of oxygen when the eggs are replaced into sea-water. But the increased consumption of oxygen also does not rise to its normal value in the developing egg while it is in the acid solution.

(4) The butyric acid penetrates the surface and reaches the substances which react when the eggs are replaced into sea-water to cause the development of osmotic pressure below the fertilisation membrane.

(5) Changes proceed which prevent the extrusion of the membrane when the eggs are returned to sea-water if they are allowed to go on for more than three or four minutes. These changes are reversed if KCN is present in the sea-water to which the eggs are returned after the acid treatment.

(6) When the eggs are returned to sea-water

- (1) the increase of permeability takes place;
- (2) the increase of oxygen consumption reaches its full value;
- (3) membrane-formation occurs if:
 - (a) the penetration of the fatty acid is sufficient;
 - (b) the sea-water is not less alkaline than pH 7.3-8.1;
 - (c) the changes mentioned in (5) above have not gone too far.

SUMMARY.

1. The effects of alterations in the reaction of the medium upon the various changes which succeed the initiation of development in the egg of *Sphaerechinus granularis* are described. It is concluded that the absence of a visible membrane in eggs fertilised in acid sea-water is not a secondary result of any of the other known effects of acidity of the medium upon the sperm of the egg.

2. If eggs of *Sphaerechinus* are caused to develop without the formation of a normal fertilisation membrane by any of several methods, membranes cannot be formed by treatment with the usual parthenogenetic agents if more than three or four minutes have elapsed after the initiation of development.

3. If eggs in which development has been initiated in this way are placed in water containing small quantities of KCN before the appearance of the gelatinous layer (20-30 minutes after the initiation of development) and treated with substances that form membranes in unfertilised eggs, typical membranes are formed. The suggestion is made that the KCN produces this effect by inhibiting the increased oxidations in the egg.

4. It is concluded that the substances which are responsible for the extrusion of the membrane remain unchanged in the surface of these eggs after the initiation of development and therefore play no part in the initiation of development.

5. Evidence is brought forward that treatment which can only be external to the egg, namely treatment for 10 secs. with sea-water acidified to *pH* 4.4 by the addition of HCl, produces the initiation of development in a certain proportion of the eggs.

It is concluded that the primary change in the initiation of development must consist of a change of the surface conditions of the egg, while in artificial parthenogenesis membrane-formation is due to action of the parthenogenetic agent below the surface.

6. Evidence is brought forward to show that this primary surface change occurs in parthenogenetic activation when the eggs are placed *into* the solution containing the parthenogenetic agent and reasons are given why its effects do not appear in this solution.

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ON THE EARLY DEVELOPMENT OF THE ECHINODERM EGG

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III. A NOTE UPON THE RELATION OF PARTHENOGENESIS TO HAEMOLYSIS.

If it is accepted that the haemolysis of blood-corpuscles is due to a chemical effect of the haemolytic agent on the substances of the outer layer of the cell, the striking agreement between the list of substances which cause haemolysis and those which cause artificial parthenogenesis (Gray, 1916) is a strong argument that their action in causing parthenogenesis is also due to chemical combination with the substances of the egg surface.

It has been the conclusion of the preceding paper that the initiation of development is due primarily to a change of the surface conditions of the cell and not to chemical combination of the parthenogenetic agent with some substance below the surface and that membrane-formation is due to chemical changes below the surface of the cell caused by penetration of the parthenogenetic agent. But all parthenogenetic agents cause the primary surface change and the question arises why, if this change occurs on the surface itself, precisely those substances which are haemolytic agents and are especially characterised by their lipoid solubility and therefore by their power of penetrating the surface should be those which cause this change. That some other substances may also do so is shown by the experiments with HCl described above, but it is in general true that the specific parthenogenetic substances are also haemolytic agents.

With the object of discovering the relation of membrane-formation to the primary surface change in eggs activated with these substances, some experiments were performed with the eggs of *Sphaerechinus* on the effect of abnormal acidity of the sea-water on their action. The anaesthetics, chloroform, benzol, xylol, toluol, and such specific agents as saponin were used.

It has been stated in the preceding paper that the pH limit of membrane-formation, in the case of eggs treated with these substances has about the same value as in the case of eggs treated with butyric acid or fertilised with sperm (pH 7.3 to 8.1). Eggs, which have been treated with haemolytic substances in acid sea-water for different periods and have formed no membranes, will, after later treatment with hypertonic sea-water, divide and develop if cytolysis has not proceeded too far in the solution containing the haemolytic substance. It is easy to show this with the substances whose action is less violent, such as toluol or benzol, but with chloroform or saponin cytolysis follows so rapidly upon the primary surface change that it is difficult to obtain conclusive results. Only one successful experiment was performed with chloroform.

Experiment.

Some eggs were placed in sea-water shaken with toluol and diluted with an equal quantity of sea-water; the reaction was brought to pH 7.1 by addition of $N/10$ HCl. After different periods in this solution they were placed in sea-water for 15 minutes and then treated with hypertonic sea-water (50 c.c. sea-water + 8 c.c. $5/2$ N NaCl) for $2\frac{1}{2}$ hours. No membranes were formed.

Three hours later the percentage of eggs which had divided or cytolysed was estimated.

No.	Period in toluol solution	Divisions %	Cytolysed %
1	10 secs.	3	0
2	20 "	8	0
3	30 "	7	0
4	45 "	12	0
5	1 min.	30	0
6	1½ mins.	25	5
7	2 "	60	15
8	3 "	50	25
9	4 "	30	50
10	5 "	10	75
11	6 "	5	90
12	8 "	2	95
13	10 "	0	100
14	15 "	0	100
15	Eggs treated with hypertonic sea-water only	2	0
16	Eggs placed in the sea-water used in the experiment	1	0

Similar results were obtained with benzol.

That membrane-formation is due to penetration in the case of these substances as in that of butyric acid might be expected and is rendered probable by the following observation. Some eggs were treated with sea-water made acid with HCl and of about the same acidity as the usual butyric acid solution. No membranes were formed when these eggs were replaced in sea-water. But if this acid solution contained a haemolytic substance such as toluol, a certain proportion of the eggs formed membranes. Here the haemolytic substance had replaced the butyric acid which we know from Loeb's work (1913) causes membrane-formation owing to its penetration of the surface. It seems probable that the haemolytic substance causes membrane-formation in the same way.

From the above and similar experiments it is clear that the haemolytic substances can cause the primary surface change in the absence of membrane-formation. It seems therefore that in these cases also the two phenomena are distinct. The question arises whether the primary surface change is in these cases caused by a change of the surface conditions presumably due to adsorption or to some action of the substance below the surface as in the formation of the membrane.

In this connection it is interesting to enquire whether these substances act by penetration or by surface action in the haemolysis of blood corpuscles.

Ponder (1922 *a* and *b*) states that this question is still open with regard to

saponin and the bile salts and brings forward evidence to show that these substances act (a) by producing a lowering of surface tension and (b) by attacking the envelope. For the case of the bile salts he states (1922 a, p. 101):

"It appears obvious that the explanation of haemolysis by sodium glycocollate on the grounds that the salt dissolves the surface of the corpuscle is inadequate.... A more probable explanation is one which is based on changes of surface tension: possibly the solvent action of the salt plays a subsequent part."

Such a conception is very similar to the double action suggested here for the action of the parthenogenetic agent on the egg.

It cannot therefore be excluded that in cases of parthenogenesis caused by these substances the action of the parthenogenetic agent in causing the primary surface change is due to changes induced on the surface and is parallel to that part of their effect on the blood corpuscles which results in a lowering of surface tension. But there still remains the fact that it is chiefly, though not solely, the peculiarly lipoid soluble bodies which cause the primary surface change. It does not seem impossible that a change of the surface conditions of the cell might be caused as well by changes within the surface as by changes of the conditions outside it. Therefore, if it were shown that these substances cause the primary surface change by penetration below the surface, this would not disprove the suggestion that the change is itself a change of the surface conditions. It seems impossible that treatment with hydrochloric acid for 10 seconds could cause any other change in the cell than a change of the surface conditions.

Hence it seems safest to conclude that the primary surface change consists in a change of the conditions of the surface of the cell which may be produced by treatment which can only be external to the cell but which may perhaps also be caused by changes below the surface.

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ON REMAINS OF EXTINCT PROBOSCIDEA IN THE
MUSEUMS OF GEOLOGY AND ZOOLOGY IN
THE UNIVERSITY OF CAMBRIDGE

PART I. *ELEPHAS ANTIQUUS*.

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(Read 3 March 1924.)

(With Six Text-figures and Plates VI—X.)

IN the collections of the Museums of Geology* and Zoology at Cambridge there is a large number of Proboscidean remains from Cambridge and its surrounding districts as well as from other parts of England. While some of these specimens have been long known and date from the times of Falconer and Leith Adams others are more recent acquisitions which have hitherto not been noticed or described. The present account is concerned only with those forms referable to *Elephas antiquus*, those referable to *E. meridionalis*, *E. trogontherii* and *E. primigenius*, of which last species there is a very large collection from local sources, being reserved for a future paper.

The inter-relation of the various "species" which together make the genus *Elephas* has always been obscure and is still a matter for dispute, and one which is not likely to end in general agreement until a more complete understanding of the sequence in time of the forms and a clearer conception of what characters constitute "specific" differences is attained. Neither the range of variation in forms living at any one time nor the range of variation in time, or in other words the mutations, has as yet a sufficiently large body of evidence to carry complete conviction as to the evolution of the group, although thanks to the recent contributions of such authors as Depéret and Mayet, Osborn, Soergel, Schlesinger, Zuffardi and others certain ideas, some of them conflicting, are beginning to crystallise out. Under these circumstances perhaps no apology is needed for adding to the already large bulk of literature on the subject a description of the specimens from Barrington, Whittlesea, Haverhill and elsewhere, since the remains from Barrington are fairly numerous, are all members of one herd, and have never as yet been noticed or figured.

ELEPHAS ANTIQUUS FROM BARRINGTON.

The specimens from Barrington are the most numerous and consist of tusks and molars, both milk and permanent, for the most part in excellent condition. The Barrington deposits, six miles from Cambridge, lie in a valley between the

* I am indebted to Professor Marr for his permission to study the specimens in the Sedgwick Museum which form the bulk of those described in this paper and to Mr Gray, the chief attendant, who collected most of the Barrington specimens. To the generosity of Mr Cardo, the owner of the Barrington pits, the University owes its fine collection not only of the elephants but of specimens of the rest of the fauna. To Dr C. W. Andrews of the British Museum, with whom I frequently discuss points relating to palaeontology, I am continuously indebted.

villages of Barrington and Haslingfield. The bone layer is found upon a deposit of chalk marl which in turn overlies the gault. It consists of a mixture of marl, loam, sand and gravel about twelve feet thick. There are two fossiliferous layers in it; an upper one, a seam of fine gravel about half-way up in which occur arctic shells with few, if any, bones, and a lower one at the bottom in which the bones occur abundantly. As to the geological horizon of this lower level there is much question and the local geological features give little or no help to its solution. Hughes (1911, 1916) placed it in the Pliocene, Lydekker (1904) in the Pleistocene. If the age of the deposit is to be settled at all the evidence must eventually come from a study of the fauna, and, if the stage of mutation of the Barrington elephant can be established as compared with other stages, such as those of Cromer, the Essex deposits and elsewhere, an approach will have been made towards the finding of the correct answer to the question.

The fauna contains the following mammals:

<i>Hippopotamus amphibius.</i>	<i>Canis lupus.</i>	<i>C. giganteus.</i>
<i>Rhinoceros lectorhinus.</i>	<i>C. vulpes</i>	<i>C. elaphus.</i>
<i>Ursus priscus.</i>	<i>Meles taxus</i>	<i>C. capreolus.</i>
<i>Felis spelaea.</i>	<i>Lepus timidus</i>	<i>Bison priscus.</i>
<i>Hyaena crocuta.</i>	<i>Cervus dama.</i>	<i>Bos primigenius.</i>

Some members of this fauna suggest a warm climate and the absence of *Elephas primigenius*, so common in some Cambridge deposits, as well as of *Rhinoceros tichorhinus* and *Rangifer* is in this respect noteworthy. Hughes (1911), in his description of the Barrington deposits, mentions the absence of the Lamellibranch *Corbicula fluminalis*, an inhabitant of warm areas and still living in the Nile, as curious seeing that it is found elsewhere in Cambridge. It has however since been found at Barrington by Mr Gray.

Of tusks there are several specimens, most of them belonging to young animals and these are only slightly curved. Two specimens are however adult and of these the one measures eight feet along the lower border with a circumference of eighteen inches at the middle part. The other is smaller, being four feet and nine inches in length and nine inches in circumference. Both tusks show a regular curve along the lower border and when seen from above a torsion represented by an elongated \S .

Of the milk teeth no specimen of the anterior one has been found. Of the second there are four specimens from the upper jaw and three from the lower. Three of the upper teeth have six plates plus a talon at each end (Figs. 1, 1 A, 2) and vary in length from 63-65 millimetres and from 31-32 in breadth. The remaining specimen has seven plates and is 67 and 36 millimetres in length and breadth respectively.

The lower teeth have either six or eight plates plus the talon at each end. The six-plated specimen (Figs. 3, 3 A) measures 57 and 25 millimetres, the other two with eight plates 78 and 34.

In all these teeth the enamel varies from moderately smooth to wrinkled and the lozenges of the sinus are variably and on the whole slightly marked. In the

specimens with the larger number of plates the anterior talon is not quite so high as in the others.

Of the third milk molar there are three specimens from the upper and three from the lower jaws of which only one upper and two lower, all associated, are complete. Of these, the specimens from the lower jaw (Fig. 4) measure 129 and

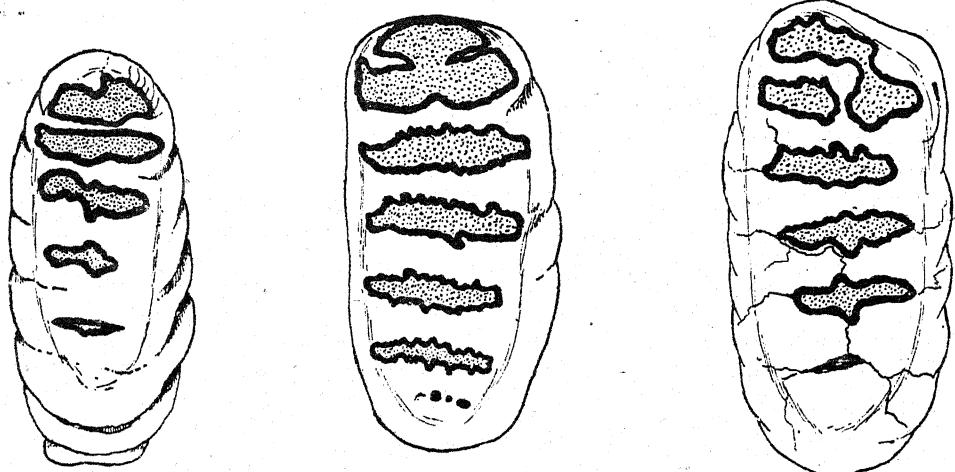


FIG. 3. Second lower milk molar. Barrington.

FIG. 2. Second upper milk molar, more worn. Barrington.

FIG. 1. Second upper milk molar. Barrington.

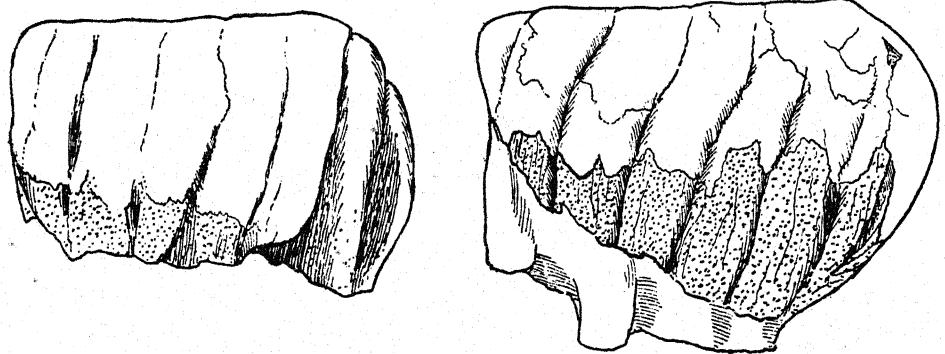


FIG. 3 A. Side view.

All figures natural size.

FIG. 1 A. Side view.

46 millimetres in length and breadth and have ten plates plus the talons. One of the incomplete specimens well worn is 53 millimetres in breadth. The corresponding upper tooth (Fig. 5) is 112 millimetres in length and 50 in breadth.

It is not easy to be certain of distinguishing the last milk molar from the first permanent one. Except in the extremes the number of plates is no guide since the last milk tooth has a range, according to various authors, of from 7-10 plates in the upper tooth and from 7-11 in the lower while the true molar runs from 9-12 in both jaws. In doubtful cases some help may be obtained by a comparison of the breadth and depth of the respective teeth but even these criteria leave one

in doubt at times as can be seen in the following description which is here interpolated of certain specimens from Haverhill which show a considerable variation in size when compared with those from Barrington.

Of these teeth there are several specimens of which two lower ones only need

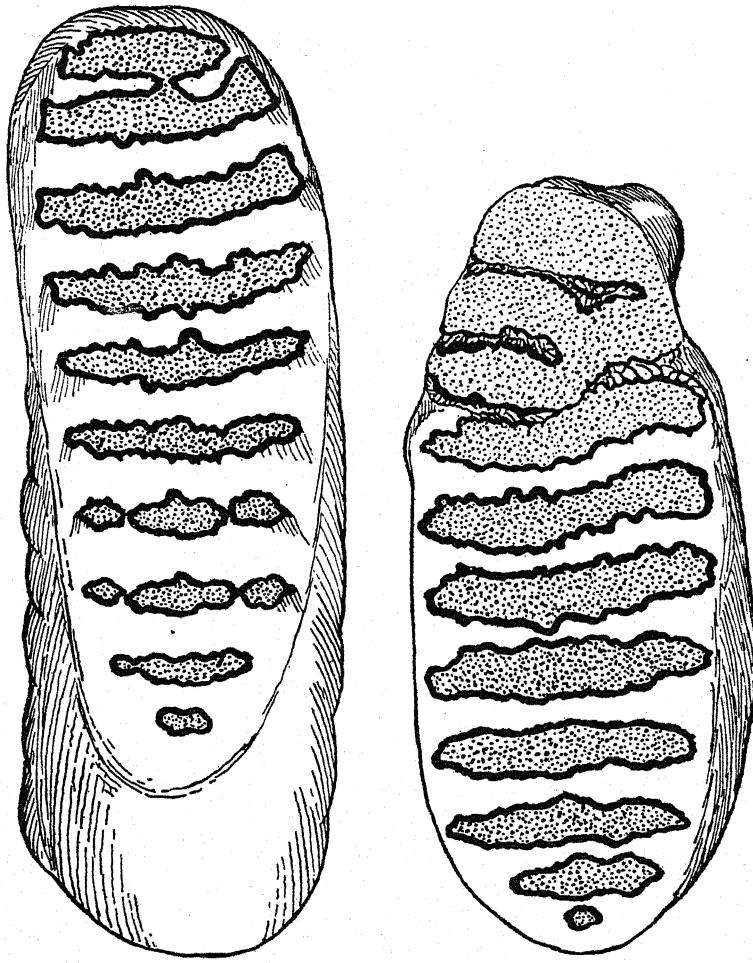


FIG. 4. Third lower milk molar.
Barrington. Natural size.

FIG. 5. Third upper milk molar.
Barrington. Natural size.

to be described to illustrate the difficulty. Each tooth has ten plates plus the talons but they differ considerably in size. The smaller of the two (Fig. 6) is 105 millimetres in length and 41 in width, the larger (Fig. 6) is 144 and 50. It is natural, and probably correct, to regard these two teeth as the last milk and first true molars respectively.

On comparing the Haverhill milk tooth with the corresponding Barrington specimens it is seen to be considerably smaller, and the presumed first molar is also rather smaller than the Barrington one.

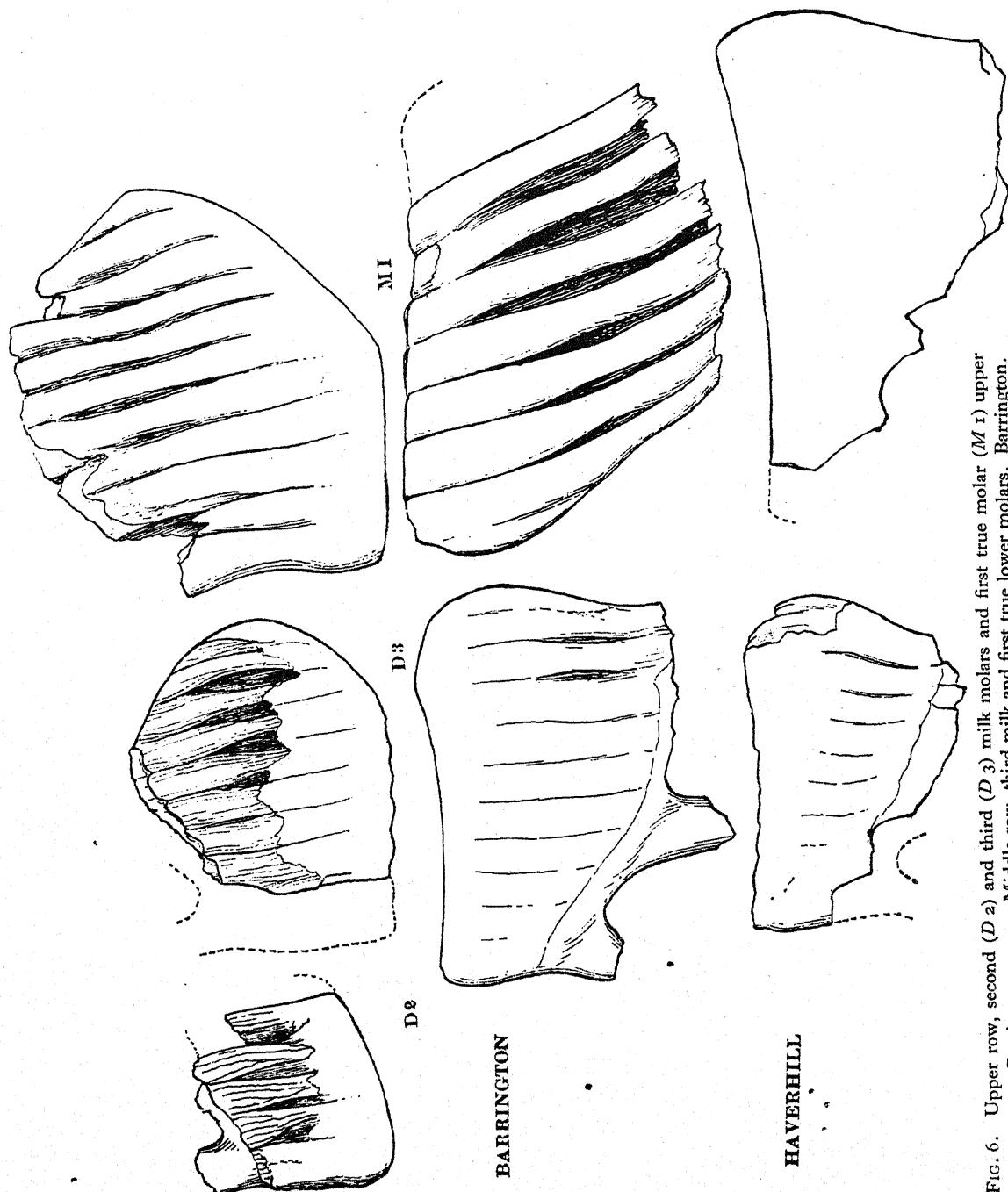


FIG. 6. Upper row, second (D_2) and third (D_3) milk molars and first true molar (M_1) upper jaw. Barrington. Middle row, third milk and first true lower molars. Barrington. Lower row, third milk and first true lower molars. Haverhill. All figures one-half natural size.

That the Barrington teeth are the last milk and not the first true molars seems probable from their association with the upper tooth (Fig. 6) which seems too small to be a permanent molar. Moreover there are in the collection fragmentary teeth of larger size but too small to be the second molars and which are to be regarded as first molars. Fig. 6 shows the outlines in side view of a second and third upper milk molar (D_1 and D_2) and a first upper molar (M_1) together with a last milk and first molar from the lower jaw of specimens from Barrington. Below these are the outlines of the lower third milk and first molar of the Haverhill specimens just referred to. As the differences between the third molars from Barrington and Haverhill are only trifling the forms may therefore be considered as of the same mutation stage of *antiquus*. They show that a certain range of size, in teeth of similar plate numbers as well as of different plate numbers (in the case of the second milk tooth), can exist in animals living at the same time.

The number of specimens upon which measurements can be made is not sufficient to give any very trustworthy idea of the range of variation but, as increase in size and in number of plates is of importance in considering the mutations in time, any information, however meagre, as to its existence or otherwise is of importance.

Of second molars there is unfortunately only one that is moderately complete; in a length of 134 and a breadth of 58 millimetres there is a posterior talon and ten plates to which must be added either one or two which have been worn away in front.

Of the third molar, which is the one most generally used for comparison, there are several examples which are sufficiently complete to afford accurate measurement.

Four absolutely complete specimens of the lower molar show a range in overall length from 265 to 300 millimetres. The ridge formula is in all cases seventeen plus the talon at each end, and making a probable allowance for missing plates. In the other slightly worn molars this seems to be a very constant figure. In all the third molars the index of laminar frequency (the number of ridges in a standard length of 10 centimetres) is from six in the well worn area up to seven and a half or more in unworn parts. The general average may be taken as seven plates. The lozenges, or median expansions of the plates, are present but not strongly emphasised and are more noticeable when the plate is somewhat worn. The condition of the enamel which is rather thin and finely wrinkled is best seen by reference to the plates. The general measurements are given in Table I in comparison with teeth from Haverhill and Whittlesea. Typical specimens are illustrated in Plates VII and IX, figs. 1 and 2.

MOLAR TEETH FROM HAVERHILL.

From Haverhill, in Essex, just over the border of Cambridgeshire, in addition to the milk molars already mentioned was found a small but well-preserved set of adult teeth, of which an associated set consisting of a second and third upper molar and third lower molar of one side are illustrated (Pl. I) in side view, and the corresponding second and third lower molar of the opposite side are

illustrated (Pl. X, fig. 1) from the crown surface. The measurements of these teeth, which differ only in the smallest particulars from the Barrington forms, are given in Table I.

FRAGMENTS FROM NEWMARKET.

These are too broken and fragmentary to allow of detailed description but as far as comparison can be made are of the Barrington form.

MOLAR TEETH FROM WHITTLESEA.

From Whittlesea in the fen district have been found a few specimens of *E. antiquus* which are very different from those already described. They consist of a lower jaw with the two last molars in place (Pl. VIII) and a separate lower third molar (Pl. X, figs. 5 and 6) which are in the Sedgwick Museum of Geology and an incomplete third lower molar in the collection of the Museum of Zoology (Pl. X, fig. 3).

These specimens have, most of them, already been referred to by Leith Adams (1877, p. 178) but have never been figured and need some further comment. The separate lower molar in the Sedgwick Museum bears a label which states that it was "found resting on surface of Oxford Clay at base of marine gravel pit close to railway station 1886, intermediate between *antiquus* and *meridionalis*" (this specimen was found after the publication of Adams' monograph). It is probable that the other specimens were found at a similar level. Adams' statement concerning them is that "the thick plated tooth which puzzled Falconer at first and caused him to correlate it with *E. priscus* of Goldfuss, until further instances showed it to be only a variety of *E. antiquus* and of the broad and thick plated crown described at page 33" (this refers to Adams' variety C, typically represented by a tooth from the valley of the Thames at Culham which he was unable to figure), "is further illustrated by two noteworthy examples I have examined lately. They were dug up at Whittlesea mere and are now in the Museum of Zoology. These two lower molars are not quite entire...are quite undistinguishable from their colossal companion" (i.e. the Culham specimen) "...the enamel being also deeply crimped, with the usual central expansions and angulations of the crown of *E. antiquus*. For comparison with these interesting molars there is a portion of a mandible from Whittlesea in the Woodwardian collection...etc." [= the Sedgwick Museum of Geology]. Those specimens referred to are now figured here.

With the foregoing brief description of the Barrington and Whittlesea teeth and by reference to the figures we may now try to place them somewhere in the series of *antiquus* mutations.

To do this it may be useful to review briefly the ideas of certain other authors as to what *antiquus* is and as to what are its mutations. Falconer (1868), who founded the species, unfortunately did so upon the characters of a number of specimens without making any particular one of them the type. There is, however, in the Sedgwick Museum a third upper molar from an unknown locality of which Falconer stated that "it is quite perfect, consisting of sixteen ridges, of which the anterior eleven are more or less worn. The discs of wear are expanded and opened,

with thick boldly crimped enamel, and exhibit all the characteristic marks of the species in the most typical way. The specimen is probably the most perfect remain of a last true molar in the kingdom" (*Memoirs*, vol. II, p. 182. The italics are the present writer's). Under the circumstances and although finer specimens have since been found in plenty the specimen is here figured, for the first time, as a sample of what Falconer considered typical, Pl. IX, fig. 3. Most authors seem to agree in considering the larger forms as typical, at all events by implication.

Table I.
Comparative measurements in millimetres of third molars.

	Length	Breadth	Depth	Breadth of widest ridge	Ridge formula	Fre- quency	Plates in use	
LOWER MOLARS								
					<i>Barrington</i>			
Pl. X, fig. 2	310	79	135	66	$t + 15 + ?$	6-7½	14	
Pl. VII	290 +	72	126	64	$? + 17 + t$	7	9	
	290	71	In man- dible	59	$t + t$	7½	13	Posterior talon not formed
	260 +	85	120	71	$t + 13 + ?$	6½	13	
	305 +	74	106	67	$t + 17 + ?$	7	13	Anterior talon absent
					<i>Haverhill</i>			
Pl. X, fig. 1	310	74	128	64	$t + 17 + t$	7	10	
	290 +	76	128	65	$? + 16 + t$	7	12	
					<i>Whittlesea</i>			
Pl. VIII	340	70	150	63	$t + 17 + t$	5½	13	
Pl. X, figs. 5 and 6	384	79	141	71	$t + 17 + t$	5	16	
Pl. X, fig. 4	336 +	89	178	76	$? + 12½ + ?$	5	7 + ?5½	missing
UPPER MOLARS								
					<i>Barrington</i>			
Pl. IX, fig. 2	279	76	183	67	17	7	9	Talons not developed
					<i>Haverhill</i>			
Pl. VI, fig. 2	220 +	72	175	61	$14 +$	—	5	
	—	78	—	69	$? + 15 + ?$	7	7	Enamel thick

Depéret and Mayet in a recent publication (1923 a) have arrived at very definite and interesting conclusions as to the inter-relationship, or rather the lack of it, in the genus *Elephas*. According to them there are five absolutely separate lines, each containing one or more species and mutations, as follows:

- I. *Primitigenius* line: *astensis*, *primitigenius*, *trogontherii*, *sibericus*.
- II. *Meridionalis* line: *planifrons*, *hysudricus*, *meridionalis*.
- III. *Antiquus* line: *ausonius*, *antiquus*; *melitensis*, *creticus*, *cypriotes*, *atlanticus*.
- IV. *Indicus* line: *indicus*.
- V. *Africanus* line: *africanus*.

In another paper Depéret and Mayet (1923 b) lay down certain "laws" applicable to all animals which will have to be taken into consideration particularly with reference to the Barrington specimens. These laws are:

Increase in size. In any line of animals the later forms should be larger than the earlier.

Progressive specialisation. As the members of a line proceed in time the various organs become more and more specialised up to the point of extinction.

Irreversibility of evolution (Dollo's law). The change implied in the first two laws cannot be reversed. An animal, that is to say, cannot become smaller or less specialised than its ancestors.

While there is no doubt that these laws in the main represent known facts it cannot be that there are not exceptions. The authors consider, however, that the pygmy elephants of the Mediterranean represent a separate primitive group and not secondarily dwarfed forms. On the other hand it cannot seriously be maintained that the Shetland pony is a primitive horse, nor the pygmy hippopotamus an ancestral form. The clear separation of lines in the genus *Elephas*, if it can be proved, will certainly help our understanding of the group though it only postpones the difficulty of finding the common ancestor to some level further back in time, and it must be remembered that other authors have considered, and still consider, the elephants as having other and closer relationships to one another. Nor do they see such clear distinctions between species as these lines indicate. Leith Adams, for instance, says that "to attempt to draw a sharp line between one species of elephant and another is impracticable in several instances; for example, although the ordinary true grinder of the mammoth, *E. antiquus* and *E. meridionalis*, can be easily distinguished when entire and the crown sculpturing fully developed; still there are varieties of crowns in these and other species barely distinguishable from one another" (Adams, 1877, p. 3).

Schlesinger (1912) derives *antiquus* from *stegodon* via *priscus*; Soergel (1912) derives it from *stegodon* via *planifrons* and *meridionalis*. Depéret and Mayet, as we have seen, derive it from an earlier mutation *ausonius* and admit of no connection with other forms—except a remote one with the Mediterranean small species—and do not commit themselves to any ancestry except that it, and the others, appeared at the beginning of the Pliocene as a sudden migration from an unknown source.

As Soergel derives *antiquus* and *trigontherii* as diverging forms from *meridionalis*, the first to become extinct without successors and the second to lead into *primigenius*, it follows that, if his view is correct and the material of his paper requires that his views should be considered with care, there must have been a stage in which the two forms were barely distinguishable. For this reason, although the position of the specimens treated in this paper have not so much bearing on the question as the forms of *trigontherii* which are to be dealt with later, a list (Table II) is given of characters, according to several authors for *meridionalis* and *trigontherii* as well as for *antiquus* and its chief described forms.

The Barrington and Whittlesea forms clearly come under the general heading of *antiquus*—there is no question, that is to say, of confusion with *meridionalis* on the one hand (in spite of the museum label already quoted) nor of *trigontherii* on the other, as may be seen by reference to the table. It is here also unnecessary to give the characters of *primigenius* or of those forms of *antiquus* from the Mediterranean and elsewhere which are sufficiently distinguished by the lesser number of plates in the third molars.

TABLE II

<i>meridionalis</i>	<i>antiquus</i> mut. <i>australis</i>	<i>antiquus</i> "type"	<i>antiquus</i> var. <i>trigontheroides</i>	<i>trigontherii</i>
Number of plates in the teeth, excluding talons, according to Zuffardi	3 5/6 7/8 8/9 8/11 10/14 9/11 11/14	3 5/7 8/10 9/12 12/13 15/20 6/8 10/12 16/21	Same as <i>antiquus</i>	Where single figures are given, it means that the number of plates is the same in the upper and lower molars.
Depéret	3 6 7 9 9/10 11/13 7/8 8/10 9/11 11/14 and mutations up to 11 11/12 12/15 11/12 11/13 12/16	2 5/6 8/10 9/12 10/14 15/20 3 6 10/12 11/15 16/21	4 6/8 9/12 9/15 14/16 18/29 13/16 16/22	Soergel describes many intermediate forms between <i>antiquus</i> and <i>trigontherii</i> with corresponding variations in plate numbers, etc.
Soergel	3/4 5/6 7/8 7/9 8/10 11/15 3 7/10	2/3 5/7 7/10 9/12 10/13 14/19 5/8 7/11	See note at side	The capital letter in brackets means that the statement is taken from Zuffardi, Depéret or Soergel.
Lamellar frequency, i.e. number of plates on moderately worn surface which lie in a standard length of 10 cm.	4 1/2 according to mutation [D]	5-6 [D]	5 1/2-7 1/2 [Z]	6-8 [D]
Thickness of enamel, its folding, sinuses or lozenges, schmelzfiguren [S], etc.	Very thick plates broad with many coarse indentations which however may be regular in the middle [S]. Thick and little folded, varies according to mutation, lozenges variable [D]	As <i>antiquus</i> but more deeply folded, and with folds more numerous, a very regular feature. Loxodont sinuses variable but the whole stronger and more prominent [D]. Elongated straight crowns about 1/3 smaller than <i>antiquus</i> from Clacton, Saffron Walden, etc. [D].	Thick, much folded plates regular rhombic or rectangular [S]. Fairly thick, always folded. Loxodont sinuses always well marked [D]. Folding not particularly noticeable, lozenges marked, a considerable difference in size between upper and lower molars [Z]. Long straight high crowns [Z].	Enamel varies, plates regular, broad and band like, the middle often suddenly swelling out, sometimes rhomboid [S]. Plates numerous, compressed with parallel enamel bands without lozenges [D]. Short broad crowns moderately high [Z]. An early mutation of <i>trigontherii</i> [D].

The main comparison therefore must be made with *ausonius* and with whatever may be considered as the typical form of *antiquus*.

Ausonius has been fully described and admirably figured by Depéret and Mayet (1923 a, Pl. X, figs. 1 and 2) and its distinguishing characters according to the authors are:

(1) A deeper folding of the enamel, and with more numerous folds. This is quoted as a very constant character.

(2) Loxodont sinuses though variable on the whole more prominent than in *antiquus*.

(3) Crowns of molar teeth elongated, straight and about one-third smaller than specimens from Clacton, Saffron Walden, Taubach, etc.

Otherwise the characters agree in number of plates and laminar frequency, etc., with *antiquus*.

It is clear that the Barrington and Haverhill teeth cannot be considered as equivalent to those of *ausonius* because:

(1) The enamel is not particularly deep, nor are the folds very numerous*.

(2) The loxodont sinuses are not remarkably prominent.

(3) They are proportionally as much smaller than *ausonius* as *ausonius* is smaller than the largest specimens of *antiquus*. The measurement of *ausonius* is taken from Depéret and Mayet's figure already quoted which is reproduced one-half size and so may be directly compared with the figures given in the present paper.

(4) The laminar frequency is seven, or even a little higher. This is a considerable difference from all other *antiquus* forms which are from five to six except var. *trigontheroides* of Zuffardi with which form the present specimens have little else in common.

According to the first of Depéret and Mayet's laws quoted above the Barrington and Haverhill elephants, being considerably smaller than the type of *ausonius*, should be by so much the earlier, and as *ausonius* is to be considered as of the Upper Pliocene period they should be considered as somewhat earlier. On the other hand, if we assume that a higher index of laminar frequency is a more advanced condition than a lower one, and this seems to be a rule for the genus *Elephas* as a whole, then the forms under discussion must be considered as later than *ausonius*, so that either the first or the third law must be broken. It seems therefore that they cannot be placed as representing a mutation in a direct line, whether in front or behind, with *ausonius*.

Turning to the Whittlesea specimens it will be seen that they agree in general terms with a specimen from Clacton (Pl. X, fig. 4, British Museum, 27907 a, already figured by Leith Adams, *loc. cit.*, Pl. IX, figs. 1, 1 a. This figure was kindly selected for me, and the photograph made, by Dr C. W. Andrews as a typical specimen from Clacton). There is however a considerable variation in size. The smallest (the specimen *in situ* in the jaws, Pl. VIII) is smaller than the type figure of *ausonius*. The next is the separate molar in the Sedgwick Museum which

* The folding and condition of the enamel are shown life size in the figure of a fragment of a molar from Haverhill on Pl. I, fig. 3.

is distinctly larger than *ausonius* and finally the damaged tooth in the Museum of Zoology which is very much larger and when an allowance is made for five and a half plates (to bring the number up to seventeen) its size would not be far short of the largest known forms. But Depéret and Mayet make the statement that *ausonius* is one-third smaller than the later mutation and cite Clacton as a level where the later mutation occurs. A Clacton form is shown here which is almost *ausonius* in size, and, except for a little more curvature and perhaps a little less wrinkling of the enamel, not very different from it. Since from the one place in Whittlesea three forms are shown varying in size from below to much above *ausonius* and one of them at least showing similar wrinkling and enamel it appears that here were several mutations living side by side.

SUMMARY.

It appears that the Barrington and Haverhill *Elephas* is somewhat distinct from other forms of *antiquus* by its higher laminar frequency. As far as is known it was a form with small molar teeth. The fact that there is no great variation in size may be due to the small number of specimens measured but is none the less interesting. The light they throw on the geological level of the beds in which they occur is obscure, but on the whole the period seems to be early rather than late and may be placed provisionally near the top of the Pliocene.

The Whittlesea forms appear to be equivalent in their general stage of evolution to those from Clacton, etc. Even in the few specimens discovered there is a wide range of variation in size which would include *ausonius* at one end and approach the largest and latest forms of *antiquus* at the other. Certain of them however are not unlike *ausonius* so that the value of this form as a time mutation appears to be somewhat impaired.

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DESCRIPTION OF PLATES.

Unless otherwise stated all the specimens figured are in the Sedgwick Museum of Geology.

PLATE VI.

FIG. 1. Third lower molar.

FIG. 2. Second and third upper molars.

FIG. 3. Portion of a molar, surface view.

All specimens from Haverhill. Figs. 1 and 2 are reduced to one half. Fig. 3 is natural size.

PLATE VII.

Lower jaws from Barrington with worn portions of the second molars and the third molars partly in wear. Half size.

PLATE VIII.

Lower jaws from Whittlesea with third molars *in situ*. Half size.

PLATE IX.

FIG. 1. Second upper molar from Barrington.

FIG. 2. Third upper molar from Barrington.

FIG. 3. Third upper molar described by Falconer as typical of *Antiquus*. Origin unknown.

All figures half size.

PLATE X.

FIG. 1. Third lower molar and part of second from Haverhill.

FIG. 2. Third lower molar from Barrington. (Museum of Zoology.)

FIG. 3. Part of a third lower molar from Whittlesea. (Museum of Zoology.)

FIG. 4. Third lower molar from Clacton. (British Museum.)

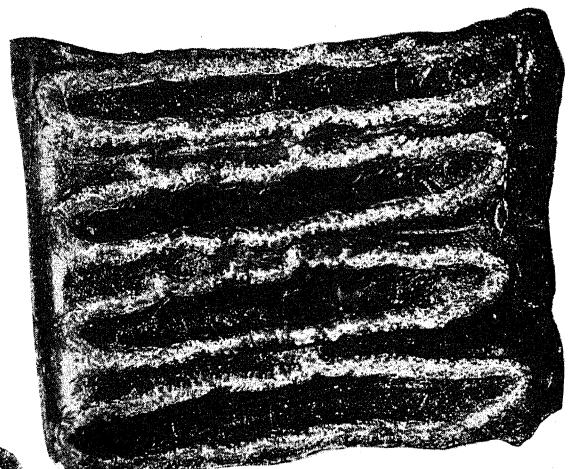
FIG. 5. Third lower molar from Whittlesea. Side view.

FIG. 6. The same, surface view.

All figures half size.



Elephas...
antiquus (Ed.)
Gravel
Haverhill, 1902



D. (Proboscidea)
DUGI (HINDAP)
SRI LANKA
Sri Lanka
Sri Lanka

PROC. OF CAMB. PHIL. SOC. (BIOLOGICAL SCIENCES)

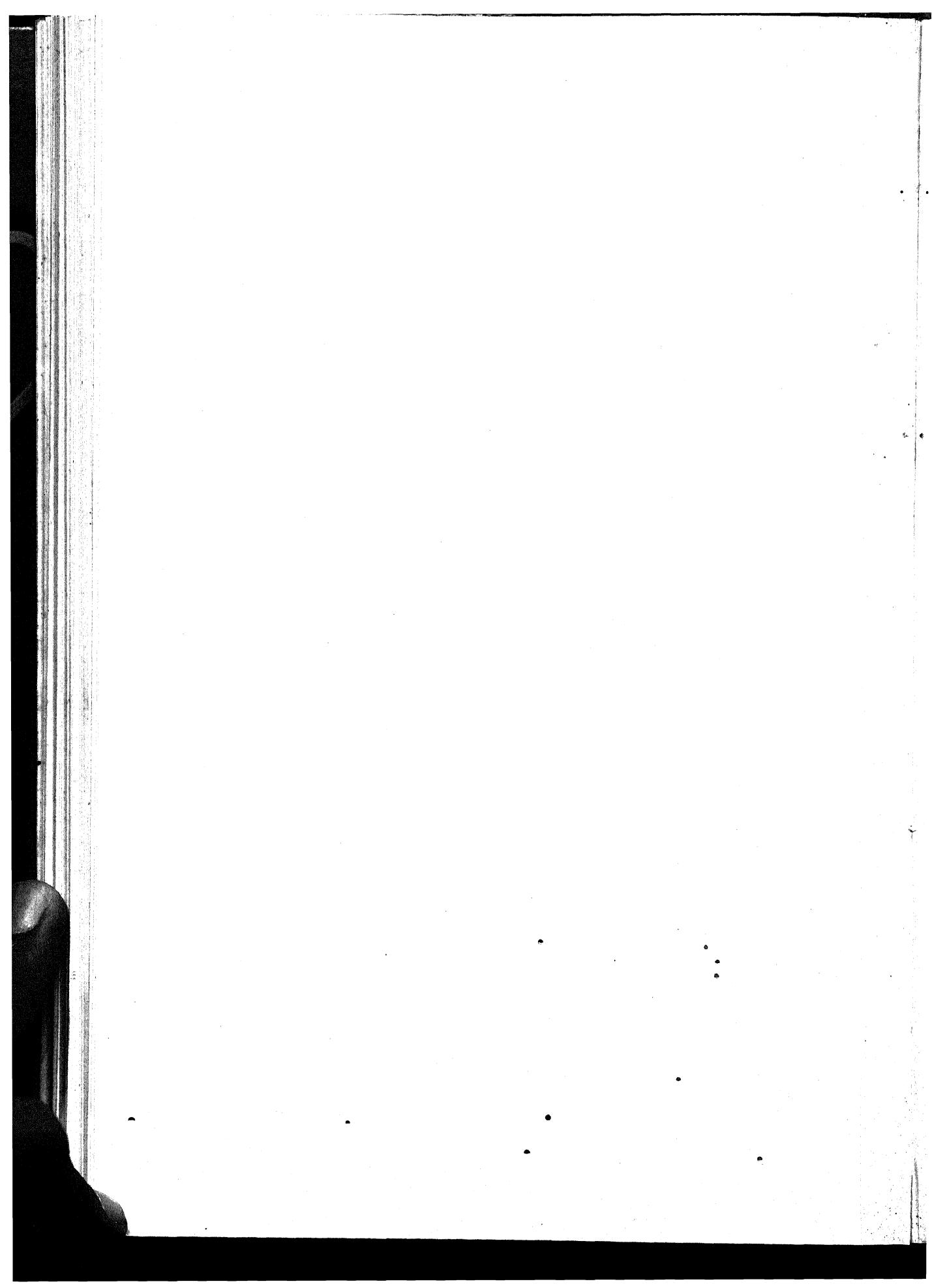


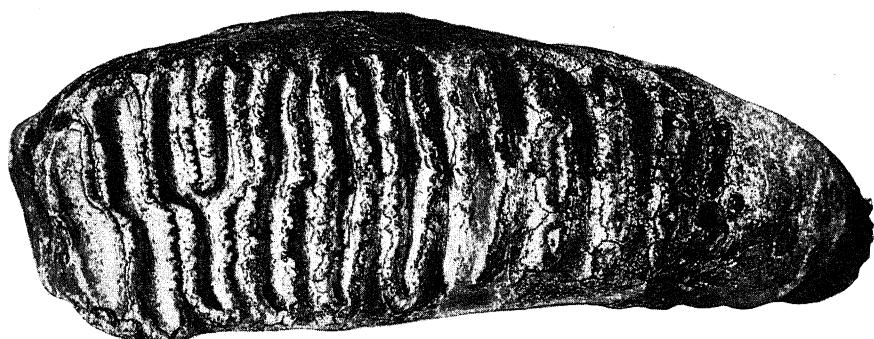
VOL. I, PLATE VII.







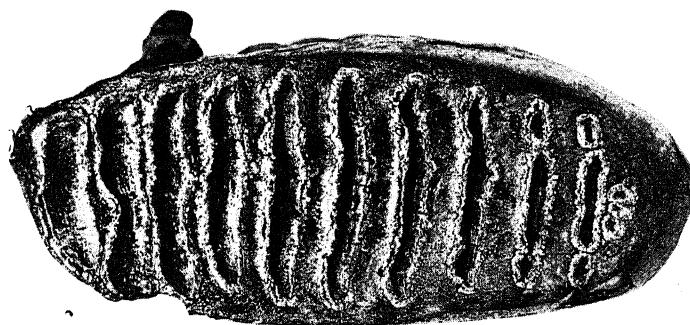




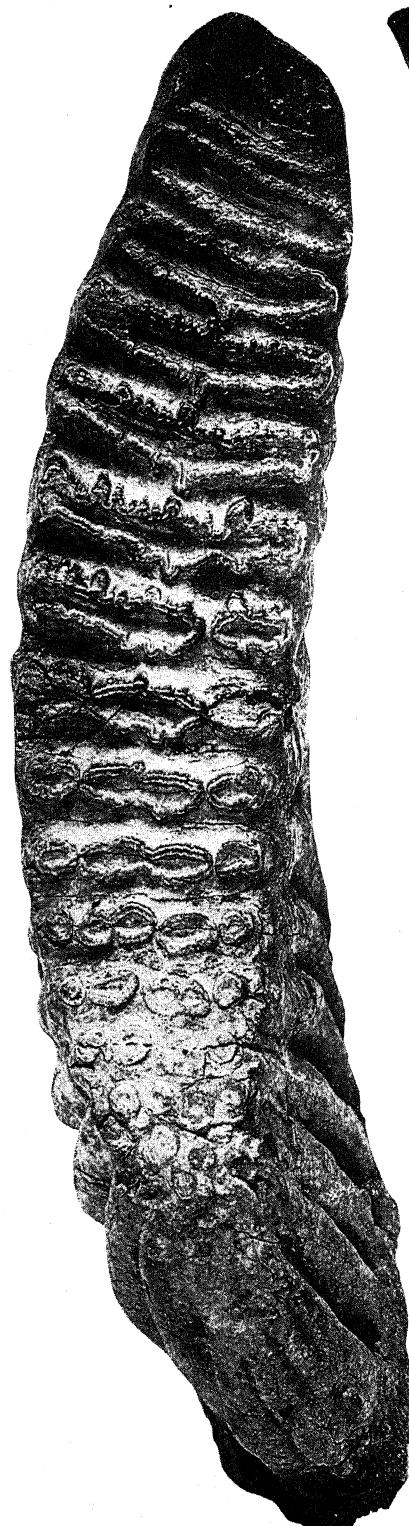
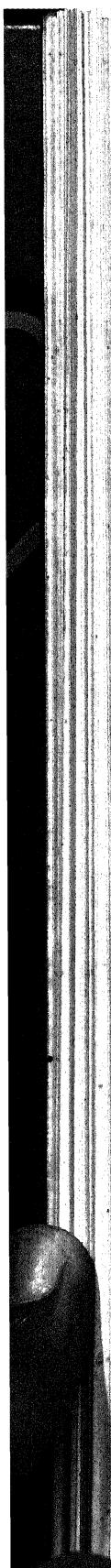
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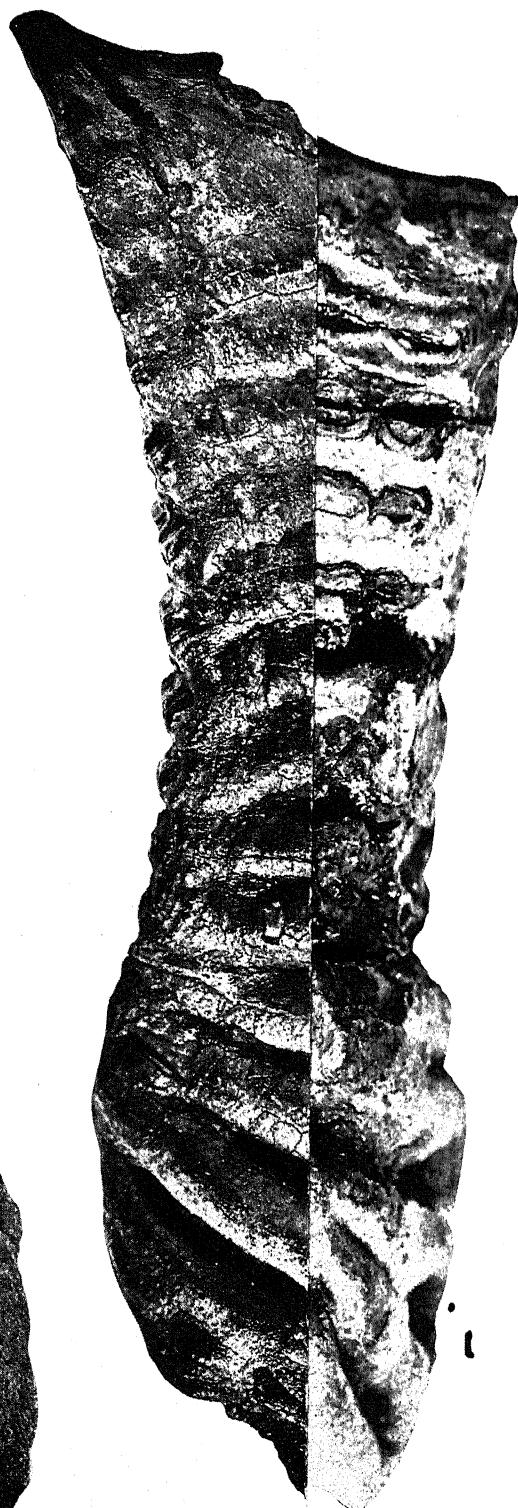
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1



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3

VOL. I, PLATE X.



A LEAF INDEX AS A HELP TO THE IDENTIFICATION OF POTATO VARIETIES

BY REDCLIFFE N. SALAMAN, M.A., M.D., BARLEY.

(Read 26 November 1923.)

(With Four Text-figures.)

THE experience gained from several years' work as Chairman of the Potato Synonym Committee of the National Institute of Agricultural Botany has shown that under certain circumstances experts whose whole life has been spent in close contact with the Potato may be baffled, and fail to recognise the most familiar and usually distinctive standard varieties or, on occasion, to distinguish any difference between an existing well-known variety and a new seedling. Thus in company with two of the most experienced potato growers in the United Kingdom, I have failed to recognise a plot of Up-to-date which had undergone a very active second growth in mid autumn, whilst on several occasions the entire Synonym Committee have experienced great difficulty in distinguishing the well-known variety Majestic from one of its own seedlings.

Considerations of this kind have led me to seek for some more or less mechanical method which could be called in aid, and it seemed not unlikely that a breadth/length index of the leaf—similar in nature to the cephalic index in man—might possibly be just such a device as was required. It was not till the enquiry had covered the examination of some 50 varieties that one found that attempts of a like kind had been made in respect to other plants. Leake*, 13 years ago, made use of a somewhat similar factor for distinguishing different varieties of cotton and found that it might behave as a Mendelian unit. Leake, however, selected typical leaves from certain parts only of the plant, and then only took two leaves from each plant. With the Potato it has not been necessary to make such reservation. Brotherton† has recently used a similar index for determining the heredity of the "rogue" character in the culinary Pea and found it to be specific for different varieties and for the "rogue," and further to be controlled by Mendelian factors.

The potato plant, however, is on an entirely different footing from either the Cotton or the Pea. For these latter, being propagated by seed, variation in the offspring may always have a gametic as well as an environmental background; with the Potato, propagated as it is by tubers, every variety, no matter to what extent it is grown, is essentially one individual, and variation within it would be—apart from the very rare possibility of somatic mutation—entirely due to environmental influences.

The method used for determining the index is to pick the leaflets from a well-grown and healthy plant or series of plants, selecting always the first, *i.e.* the one under the terminal, leaflet on the left of the mid-rib. The leaflets are placed

* Leake, H. M. "Studies in Indian Cotton." *Journ. Genetics*, 1, 205, 1911.

† Brotherton, Wilber Jr. "Further Studies on the Inheritance of 'Rogue' type in Garden Peas (*Pisum Sativum L.*)." *Journ. of Agric. Research*, 24, No. 16, June, 1923.

upon a blotting pad and covered with a thin sheet of white paper and rubbed, one by one, with a soft broad-leaded pencil. When the impressions have been taken, the original leaves can be thrown away. Satisfactory results have been obtained on leaves sent by post and "rubbed" on the second and even on the third day after plucking.

The leaflet, or rather its rubbing, is measured in two directions, its greatest breadth and its greatest length. The first measurement offers no difficulty, the latter, owing to a peculiarity of the potato leaflet, requires some consideration.

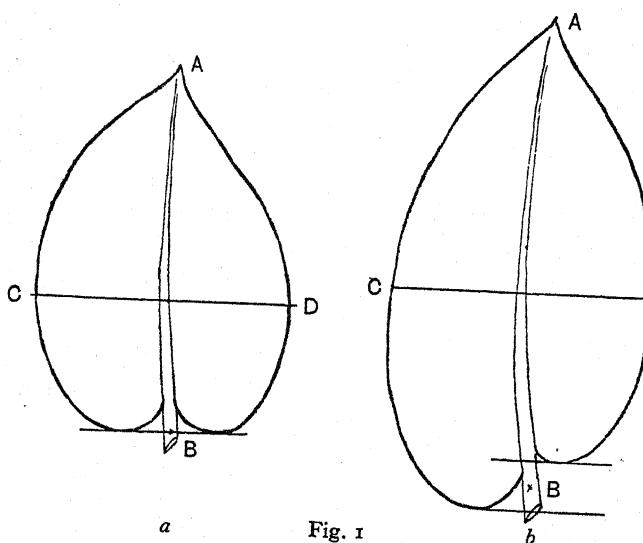


Fig. 1

A leaflet at its junction with its petiole may be either symmetrical (Fig. 1, a) or asymmetrical (Fig. 1, b). In the former case a line is drawn in contact with the lowest portion of each lateral lobe D and the spot where it bisects the mid-rib is taken as the lowest limit of the leaf. In the asymmetrical leaf, the inner half of the leaf is always shorter than the outer. Indeed,

amongst the 2994 leaf-

lets, the rubbings of all of which have been taken and preserved, there has been no exception to this rule. In such cases a line is drawn at right angles to the mid-rib from the lowest point of each lobe, and the point midway between the two lines as they intersect the mid-rib has been taken as the lowest point of the long axis for the purpose of this measurement. The actual measurement was done with a pair of dividers, and the scale used was that of the inch divided into tenths and fiftieths. Of course any scale would do equally well but this was found to be easy of use and to afford as great a degree of accuracy as was possible. The value for the axes AB, CD (Fig. 1) having been determined, the index $\frac{CD}{AB} \times 100$ was determined by the slide rule. As a result of trials which are recorded later, it was decided that the least number of leaflets upon which it is wise to base an "index" is twenty: in many cases a greater number has been used.

It may be recalled that the leaf of the potato plant is a compound one composed of a mid-rib terminating in a single median lobe and two, three, or sometimes four pairs of lateral leaflets. Between these leaflets are inserted in the mid-rib one or more pairs of smaller leaflets or folioles. In some varieties the folioles may become almost as big as the leaflets, whilst in others they remain quite small, and

in wild varieties they are often absent. It has always been recognised that the terminal leaflet varies considerably in shape from the laterals and, a fact which is of greater importance in this connection, it may frequently be deformed and not seldom more or less united with one or other of the last pair of lateral leaflets. However, this terminal leaflet has received more attention from experts than the laterals, a circumstance which has not assisted towards any clear definition of foliage type. Indeed, Potato foliages are often described as being broad or even rounded leafed, when it is only the terminal leaflet which may exhibit this character, and that not consistently.

For the purposes of this investigation a preliminary trial was made of the index of the various lateral leaflets in one variety and it was at once clear that whilst the index of right and left side were practically identical and those of the first and second generally alike, that for the third pair differed widely from those more distally placed.

Thus in Great Scot and Kerr's Pink the indices are:

Table I

		Great Scot	Kerr's Pink
1st leaflet	Left	60.8	66
1st "	Right	60.5	65.8
2nd "	Left	60.4	65.3
2nd "	Right	60.5	65.8
3rd "	Left	72.25	75.5
3rd "	Right	71.6	75

It was necessary, therefore, to fix on one definite leaflet for examination and the choice was made of the first leaflet on the left in all cases.

From the outset it seemed desirable that the leaflets measured should belong to well-grown and mature leaves, *i.e.* the lower leaves were preferred to the upper ones. In order, however, to test whether the index of a given leaf was constant during the growing season, photo-impressions on silver paper were taken of two leaves on two plants during the season. Both were very vigorous seedlings but one, "M," suffered with mosaic disease, as shown by a very definite mottling, whilst the other, "N," was normal. The results are seen in Table II.

Table II

Date	"N"			"M"		
	Length in 1/50 of inch	Breadth	Index	Length	Breadth	Index
23. viii. 23	96	59	62	74	46	62
27. viii. 23	105	66	62.5	90	55	61.5
30. viii. 23	111	69	62	94	58	62
3. ix. 23	114	70	61.5	98	59	60
9. ix. 23	113	70	62	100	60	60
24. ix. 23	—	—	—	109	65	59.5
29. x. 23	—	—	—	117	68	58

Unfortunately the "N" series is not complete as the leaf was destroyed by slugs and could not be measured any further. So far as it goes, however, the normal leaf during 18 days maintained a uniform index of 62, whilst the leaf with mosaic markings but no deformity, during a space of 68 days, suffered a loss of index from 62 to 58. At the same time if we compare it with the "N" leaf over the 18 days of the latter's existence, we find a lowering of index from 62 to 60.

Table III

Date	Variety	No. of leaves measured	Average length in inches	Index	
7. viii.	Ceres	19	3.7	53.3	Mature leaves
7. viii.	"	11	2.0	59	Secondary "
7. viii.	Evergood	20	2.8	58	Mature "
9. viii.	"	13	1.7	61	Secondary "
9. viii.	Seedling 324B2	12	2.9	65.1	Mature "
9. viii.	"	12	1.6	67	Secondary "
16. viii.	Nithsdale "	21	3.0	62	Mature "
8. viii.	"	10	2.0	66	Secondary "

The results obtained with four varieties in respect to the leaf index of their secondary leaves, Table III, shows that leaflets smaller than the one "N" measured during its life-time have a definitely higher index than do fully mature ones on the same plant, and it is probably quite safe to conclude that the normal leaf only acquires its final and characteristic "index" after it has attained a certain relative size, which may roughly be estimated to be such that its axis is two-thirds the length of the normal full-grown leaflet.

In estimating the leaf index of a variety, young leaves, as well as all secondary growth, must be avoided.

There is considerable evidence that slight mosaic mottling of the leaflet will reduce the index, as shown by comparison of the indices of the same variety when quite healthy and when so affected. It seems also probable that any lack of growth, whether it be due to disease or to adverse environment, produces a similar result. It is therefore essential that well-grown and quite healthy plants be selected for the determination of the Leaf Index.

The immediate environment in which the plant grows—so long as a reasonably normal growth takes place—seems to be without any effect on the index. A few examples are shown in Table IV.

It was also found that the immediate place of origin of the seed has likewise no effect on the index so long as the resultant plants are healthy. A striking example of this was the identity of Leaf Index for the wild variety Maglia, grown several years in pots at Messrs Sutton at Reading, and that obtained from plants derived from tubers imported this summer from Peru and grown in Barley.

In order to obtain a statistical analysis of the leaf size and its index, 400 mature leaflets of Tinwald Perfection were picked on two consecutive days from well-grown plants in Barley, and rubbings taken. The curve representing the distribution of the various indices is a normal frequency curve. See Fig. 2.

The mean value for the leaf index for this variety is 69. The probable error for one observation is 2.8, and for a group of 400 observations it is .13. The least number "n" of leaflets required to arrive at a fair estimation of the leaf index of any particular variety was judged to be such a number that the p.e. of the mean

Table IV

Date	Variety	Locality	No. of leaves	Index
20. vii.	America	Reading	7	65.4
25. vii.	"	Ormskirk	8	64.8
23. vii.	Arran Chief	Barley	24	62.8
27. vii.	" "	Ormskirk	32	63.4
23. vii.	Ally	Barley	11	59.4
27. vii.	"	Ormskirk	10	59.2
9. viii.	"	Cambridge	12	58
23. vii.	Epicure	Barley	18	60
29. vii.	"	Wisley	18	60.3
27. vii.	"	Ormskirk	9	59.2
1. viii.	Sharpe's Express	Barley	16	65.2
29. vii.	" "	Wisley	18	64.1
27. vii.	" "	Ormskirk	10	64.5

Frequency

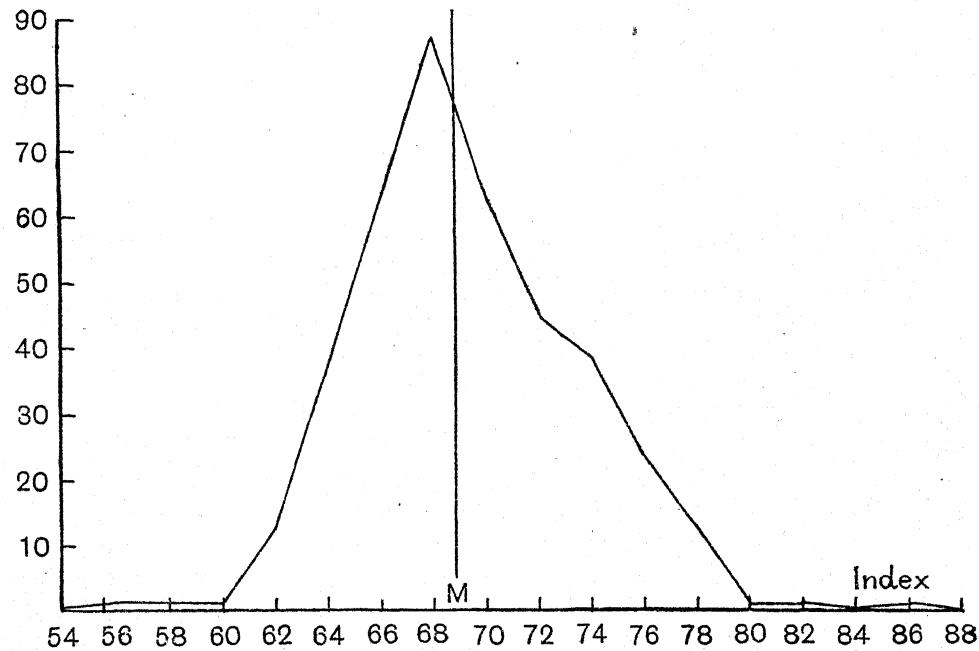


Fig. 2. Curve of Distribution of Leaf Index of 400 Leaves of the Variety Tinwald Perfection.

of "n" observations when multiplied by 3 should be well below 2. A convenient value for "n" fulfilling this condition is 20.

When all the varieties of which 20 or more leaflets have been measured are considered (Table V) it will be seen that the p.e. for a single observation in each such variety varies between 1.54 and 2.9; the average p.e. of 36 varieties in which

20 or more leaves were examined is 2.36, and the p.e. for the difference between two means each of 20 is .74*. If, therefore, the mean value of the index of the group of plants of each of which 20 leaves have been observed vary by as much as two or more units from one another, the odds are fairly large, viz. about 18 : 1, that the two groups are distinct varieties—it being always understood that the plants are healthy and the leaflets mature.

If the length of the 400 leaves of Tinwald Perfection be similarly plotted, they likewise present a frequency curve which closely approximates to the normal. See Fig. 3.

Frequency

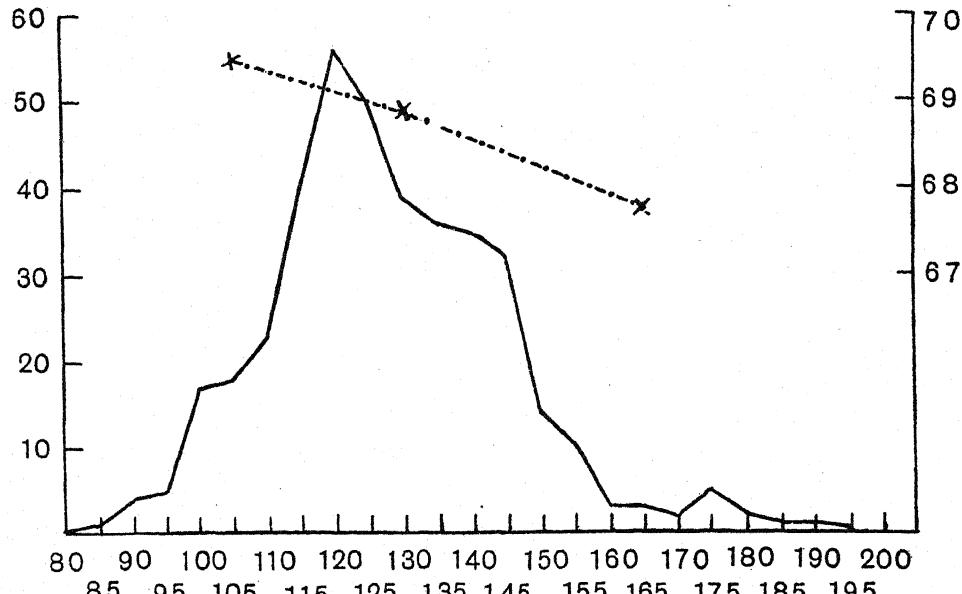


Fig. 3. Curve of Frequency of Leaf Lengths—at intervals of $\frac{5}{6}$ of inch.
—·—·—· Represents the Leaf Index, averaged about the three lengths, viz.
105, 130, 165 fiftieths of an inch.

The relation of the Index to the length of the leaf has also been worked out. The higher index of young leaflets, and leaflets from secondary growth, suggests that the index even in mature leaflets may tend to be greater the shorter the axis. The material used in this particular experiment was, intentionally, made up of mature and well-grown leaflets. Notwithstanding, however, a higher index is in this case to be observed in relation to the shorter axis. See Fig. 3. For simplicity the leaves have been grouped in three lots:

- (a) 103 leaflets with a mean axis length of 105 with average index 69.5
- (b) 218 " " " " 130 " " 68.9
- (c) 74 " " " " 165 " " 67.8

* The average was determined both as the arithmetical mean and by taking the square root of the sum of the squares of the values divided by the number of varieties. The two values were respectively 2.34 and 2.36, the latter has been used.

Table V.

Name of Variety	No. of leaflets "N" examined	Leaf Index	P.e. of "I" observation, i.e. $\cdot 674 \sigma$	P.e. of "N" observations
Abundance	40	60.9	3.2	.5
Ally	33	58.8	2.15	.38
America	15	65.1	2.15	.55
Arran Chief	56	63.5	2.8	.37
Arran Comrade	26	62.5	2.1	.4
Arran Victory	27	60.4	2.65	.51
A.V.V. 12	39	60.4	2.7	.43
Barley Bounty	45	65.2	2	.3
Bishop	15	60.4	2.23	.57
Boston Kidney	33	68.1	1.8	.31
British Queen	29	63.9	2.8	.52
Bute Blue	29	59.9	2.45	
Ceres and Triumph	34	53.5	2.5	.43
Champion II	10	48.9	3	.95
Crusader	6	57.5	.88	.36
Di Vernon	11	55.9	2.3	.7
Duke of York	18	55.8	1.9	.36
Dunvegan	8	57.2	1.34	.48
Early Manistee	20	59.6	2.15	.48
Early Market	10	63.7	1.6	.5
Edgecote Purple	10	60.8	2.6	.82
Edzell Blue	15	59	2.03	.52
Epicure	36	59.2	2.65	.44
Evergood	20	57.95	1.54	.34
Flourball	22	66.65	2.38	.5
Golden Wonder	20	60.9	2.57	.58
Great Scot	35	60.8	2.05	.34
Harbinger	9	60.9	2.6	.87
Immune Ashleaf	21	53.9	1.92	.42
Irish Chieftain	20	61.1	2.25	.49
K. of K.	29	64.1	2.9	.54
Katie Glover	25	57.2	1.87	.39
Kerr's Pink	40	66.57	2.35	.37
Kew 3 K. 23. 12	14	60.9	1.88	.5
King Edward	24	56.5	2.35	.48
King George	33	65.7	2.35	.41
Langworthy	9	60.1	2.8	.9
Lochar	10	71.5	1.96	.62
Magnum Bonum	9	57	1.56	.52
Majestic	47	61	2.3	.33
Marquis of Bute	31	66.8	2.33	.42
May Queen	24	57.6	2.57	.53
Mr Bresse	13	59.9	1.82	.5
Myatt's Ashleaf	24	57.1	2.65	.54
Ninetyfold	28	56	2.27	.43
Nithsdale	21	62	2.3	.5
Norma	29	65.8	2.07	.38
Northern Star	10	63	1.28	.4
President	10	61.8	1.9	.6
Purple Eyes	9	60.1	2.15	.71
Rector	10	62.8	1.74	.54
Rhoderick Dhu	34	65	2.52	.42
St Malo	11	63.3	2.3	.69
Scottish Champion	10	50.3	2.3	.73
Sharpe's Express	45	64.73	2.6	.38
Sharpe's Victor	10	56.1	1.94	.65
Sir John Llewelyn	19	58.1	2.55	.59
Templar	23	60.3	1.96	.41
The Dean	10	67.6	3.45	1.08
Tinwald Perfection	400	68.86	2.75	.13
Up-to-date	29	63.1	2.15	.4
Violette de Forez	10	61.7	2.15	.68
V2 x 24.15.48	31	57.5	2.75	.5
White City	17	67.3	2.05	.5
Witchhill	32	57.8	1.96	.35
Yam	12	65	2.05	.59
113B18.1	26	67	2.8	.54
346B30	18	57	2.8	.65
365B2	45	58.4	2.17	.32
366B20	37	58.8	2.75	.45

The difference between the indices of *a* and *b* is .6; between *b* and *c* is 1.1, and between *a* and *c* 1.7. The p.e. for a group of 74 is .32, so that the difference between *b* and *c* and *a* and *c* is considerably more than three times the p.e. and

Table VI

Index	First Earlies	Second Earlies	Maincrop	Lates
49	—	—	—	—
50	—	—	—	Scottish Champion
51	—	—	—	—
52	—	—	—	—
53	—	—	Ceres	—
54	Immune Ashleaf	—	King Edward	—
55	—	—	—	—
56	Ninetyfold	—	—	—
	Sharpe's Victor	—	—	—
	Duke of York	—	—	—
	Di Vernon	—	—	—
57	Dunvegan	Katie Glover	—	Magnum Bonum
	Myatt's Ashleaf	Crusader	—	—
	—	V2 x 24.15.48	—	—
58	Sir John Llewelyn	—	Evergood	—
	Witchhill	—	—	—
	May Queen	—	—	—
59	Epicure	Edzell Blue	—	—
	—	Ally	—	—
60	—	Mr Bresse	Bute Blue	Langworthy
	—	Early Manistee	Purple Eyes	Templar
	—	—	—	Arran Victory
61	Harbinger	Kew 3 K. 23. 12	Majestic	Bishop
	—	Great Scot	Abundance	Irish Chieftain
	—	—	Edgecote Purple	Golden Wonder
62	—	Arran Comrade	—	—
	—	Nithsdale	—	President
63	—	St Malo	—	Violette de Forez
	—	—	—	Northern Star
	—	—	—	Rector
	—	—	—	Up-to-date
64	—	British Queen	K. of K.	Arran Chief
	—	Early Market	—	—
65	America	—	Barley Bounty	Rhoderick Dhu
	Sharpe's Express	—	—	Yam
66	—	King George	Norna	—
67	—	Marquis of Bute	Flourball	White City
68	—	Boston Kidney	—	Kerr's Pink
69	—	—	—	The Dean
70	—	—	Tinwald Perfection	—
71	—	—	—	—
72	—	—	—	Lochar
		$\frac{816}{14} = 58.2$	$\frac{1047}{17} = 61.6$	$\frac{801}{13} = 61.6$
				$\frac{1248}{20} = 62.4$

hence significant. Indeed the difference between *a* and *b*, if we take the group to be 103, is .28, which is twice the p.e. and is, in conjunction with the other results, highly suggestive of a real difference of value.

Such differences in the mean would, however, only be obtained were a conscious selection for the largest or shortest leaflets exercised. In general there is no reason to think that the slight correlation shown to exist between greater length

of axis and low index is likely to disturb the result when the leaflets are plucked, as they come, from mature leaves.

In Table V is to be found the full list of all the named varieties, together with a few unnamed new varieties of some years' standing, which have been examined. The number of observations on which the index is based, the values of the p.e. for one observation as well as for "n" observations in addition to the Leaf Index itself are there recorded.

In Table VI, these same varieties are arranged according to their maturity, as First Earlies, Second Earlies, Maincrops and Lates. The average index for each group in Table VI shows a rise from Early to Late. In Fig. 4 the same fact is shown, but here the two early and the two late groups are united into one

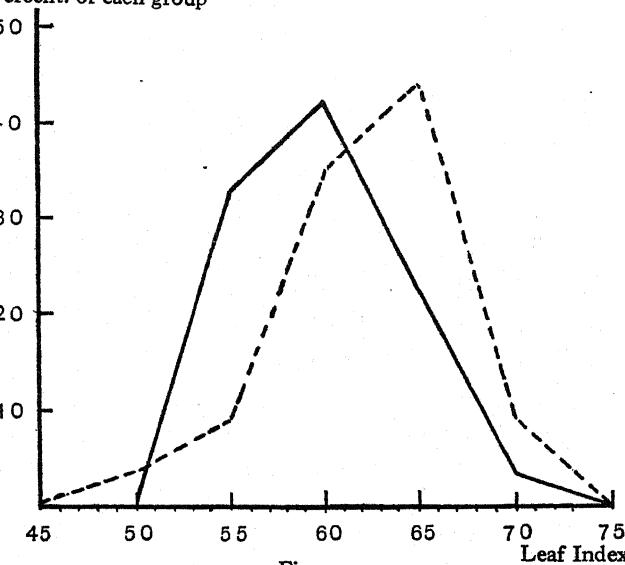


Fig. 4.
— Early varieties Total 31
- - - Late " " 34

early and one late respectively, and the percentage of each falling on the various index values is made clear. There is clearly some correlation here of a positive kind between advancing maturity and a rising leaf index. Berthault* in his excellent monograph on the Potato, classifies varieties into four groups based on foliage characters, and although it is not stated in so many words, it would appear that he recognises a positive correlation between early maturity and greater breadth of leaflet, and between late maturity and a greater length. The analysis here given of over 2000 leaflets in respect to maturity would show that although the correlation present is not of a high order, yet such as it is, it is clearly in the opposite direction to that which appears to be suggested by Berthault.

No other correlation has been observed between the physical or physiological character of the tuber or plant and the Leaf Index.

This investigation based on the possibility of varieties of the Potato having a specific leaf index was prompted by the desire to find, if possible, some sure and more or less mechanical assistance in determining the synonymy or otherwise of the various new or presumably new varieties which yearly come to Ormskirk for trial. During the 1923 season the indices of several suspected synonymous varieties were tested. In Table VII are given some of the results.

* Berthault, P. *Recherches Botaniques sur les variétés cultivées du Solanum Tuberosum*. Nancy, 1911.

In these examples, the Leaf Index of the suspected synonymous varieties approximates very closely to that of the standard variety, of which each would appear to be but a replica as regards the general appearance of their haulm and tuber.

Table VII

Name of new variety, 1923	Index	Type of which it is suspected to be a synonym	Index
No. 201	63	Arran Chief	63.5
" 237	62.8	"	63.5
" 82 a	57.7	Ally	58.8
" 179	63.5	British Queen	63.9
" 181	56.3	Ninetyfold	56
" 121	66	Norna	65.8
" 275	64.4	Up-to-date	63.1
" 63	61.1	Majestic	61
Triumph (Dutch)	53.5	Ceres (Dutch)	53.5
No. 293	64	Rhoderick Dhu	64.5

The experience of this year in regard to the use of the index as a test for synonymy has not been extensive enough to do more than point out its value in the few cases to which it has been applied, but there is some evidence that it may be of considerable value in deciding the degree of synonymy between such a standard variety as e.g. Majestic and its numerous seedlings which so closely resemble the parent.

The Leaf Index has been shown to be a constant with a small variation for the various Potato varieties; it would not therefore be surprising were it found to be controlled by hereditary factors and to behave in accordance with Mendelian law. No specific work in this particular direction has been done so far. However, two families were raised in 1923, whose behaviour in respect to the Leaf Index suggests that a very definite dominance in respect to a particular Leaf Index may occur. The wild *S. demissum**, whose index is 61, was mated to two parents: (a) a domestic seedling with an index of 57: of these, 13 F_1 plants were tested and their indices varied from 57 to 61 with an average of 59; (b) the male parent of the second family had an index of 67. Twelve F_1 plants had indices ranging from 57 to 61 as in the first family, and the average index was 58.5. Thus we have a wild variety, with an index of 61, acting in respect to two varieties, the indices of which are 61 and 67 respectively, in such a way as to produce an F_1 which is exactly the same for both families. Incidentally, the haulm, flower, and tubers, were in both families identical.

SUMMARY.

The first lateral leaflet on the left of the mid-rib of each leaf is measured, and its index breadth/length \times 100 calculated.

The Leaf Index of a variety must be ascertained from adult leaves on a healthy plant.

* There is some doubt as to whether this wild variety is correctly described as *S. demissum*.

The younger leaves where the long axis is less than two-thirds its final length will have a somewhat higher index than the normal.

Leaves of secondary growth have, at first, a higher index.

Leaves affected with slight mosaic mottling have a reduced index.

The variation of the index within any given variety is a normal one and represented by a normal frequency curve.

The mean value of the p.e. for one observation when 36 varieties are examined of each of which a minimum of 20 leaves have been measured = 2.36.

The p.e. of the difference of two means of 20 each is .74. A difference of two units in the Index may be considered as indicating odds of about 18 : 1 against the two varieties being the same, and a difference of 3 units as being almost certain evidence of such distinction, the odds being over 140 : 1.

Of 65 varieties of which the index was measured, the value of the latter varies between 50 and 72.

Neither the place of origin of the seed tubers, nor the locality where the plants are raised have any effect on the Leaf Index, always providing that the plants are healthy.

The Leaf Index is a constant for each variety.

There is some correlation between early varieties and a low index, and late varieties and a high index.

The Leaf Index has given valuable evidence as to the identity of suspected synonymous stocks growing for trial at Ormskirk.

THE PARASITISM OF *HELMINTHOSPORIUM GRAMINEUM*, RAB. (LEAF-STRIPE DISEASE OF BARLEY.) ABSTRACT

BY N. J. G. SMITH.

(Communicated by Mr F. T. BROOKS.)

(Read 26 November 1923.)

THIS fungus was considered by Ravn* to inhabit the growing point of its host, like the Smut fungi. That this might well be doubted was pointed out to me by Mr Brooks, who suggested a re-investigation of the life-history of this fungus and related forms. The results here summarised make it certain that leaf-stripe disease of barley is produced without the fungus being present in the growing-point.

The attack on the germinating seed forms the appropriate starting-point for this account. The attack occurs while the shoot is still under the adherent chaffs, or during its emergence, which usually occurs at the awn-end of the grain. The sources of the attack are (1) Conidia, mainly lodging at this end, (2) Mycelium penetrating from the chaffs, if these have been infected, while green, from the parent or another plant, (3) Perithecia, which may be formed inside or outside the chaffs. From these sources mycelium penetrates the outer surface of the coleoptile and spreads in its tissues. No invasion of other parts takes place until the first leaf, rolled-up inside, is near emergence. By this time hyphae have reached the inner surface of the coleoptile, and are there in contact with the upward-pushing first leaf. The inrolled part of this escapes penetration at this stage, as does a greater or less proportion of the exposed surface, owing to the variable amount of mycelium which has traversed the coleoptile. When the leaf expands it soon shows one or more pale stripes of invaded tissue, the stripe being in part due to the upward growth of the leaf brushing a vertical stripe against externally applied hyphae, in part to the barrier to lateral spread of mycelium provided by the vascular bundles. The second leaf is in turn infected by the enclosing sheath of the first, and similarly all leaves are infected.

Penetration of the growing-point sometimes occurs, but only *after* all surrounding sheaths have been infected. Such penetration causes death to the growing-point. Mycelium in the stem is a secondary phenomenon, the result of penetration from diseased leaf-sheaths enveloping it, and is usually left fragmentary and impotent among hardening nodes and elongating internodes. The fungus reaches the ear from its enclosing sheath at an earlier or later stage (depending on the amount of fungus which has gained a footing in the plant and on the conditions affecting the rate of spread of the fungus). Correspondingly much

* Ravn, F. K. "Über einige *Helminthosporium*-Arten." *Zeit. f. Pflanzenkr.* 11, 1, 1901.

or little chaff discoloration will result, the latter being the more dangerous as not ensuring rejection when seed grain is chosen. Much weakening of straw and awns, through similar invasion by the fungus, and "mummification" of the auricle of the last leaf, produce imperfect emergence of the ear.

Other, secondary, means of attack are not here discussed.

The host-parasite relations thus outlined clearly differ widely from those of Smut-fungi.

On the other hand, analogous phases are found in other *Helminthosporium* diseases. For example, in cases of seed-infection by *H. Avenae* and *H. teres* the stripe form of attack on the first leaf may occur. Also there is some evidence that a foot-rot of barley, similar to that caused by *H. sativum*, can be induced by *H. gramineum* under infection conditions different from those which produce leaf-stripe. The only foot-rot of barley, however, as yet found occurring naturally at Cambridge is due to *H. sativum*. Further comparison must be deferred, until further evidence is available.

SOME STATISTICAL ASPECTS OF GEOGRAPHICAL DISTRIBUTION

By A. G. THACKER.

(Communicated by Mr J. GRAY.)

(Read 21 May 1923.)

IN recent discussions on the geographical distribution of animals and plants the subject has been approached from a novel point of view. An attempt has been made to investigate certain problems of distribution by the statistical or biometrical method; and it is thought by some that this method may throw new light, not only on the phenomena of distribution, but upon the general principles of biology, of which the facts of distribution must be in some degree the expression*. There appear to be certain purely statistical aspects of the problems raised, which have so far been ignored; and since some of these considerations seem to be of a fundamental character, it is perhaps worth while to place them upon record. I advance these considerations with diffidence, because the points which I am about to make are in no way obscure; they are on the contrary very simple; and the only excuse for discussing them is that their fundamental importance for the statistics of distribution appears to have been overlooked.

The first consideration relates to a statistical comparison of the ranges of all the species in a certain area—in the simplest case, an insular area—with the ranges of the species occurring in a given part of that area. The data in such a case will be subject, apart from all biological considerations, to a purely mechanical law of probability which must apply to non-living objects and to living organisms alike. It is the most elementary of biometrical principles that the extremes of a normal curve of variation are unlikely to occur in a small sample. In a random selection of ten persons in a tube railway station we do not expect to find either an imbecile or a genius. We expect to find persons close to the mean of intelligence, because such persons are much the most numerous in the total population. It is improbable that a small sample will be truly representative of a large series; the part is not ordinarily a true representation of the whole.

And this principle can of course be applied geographically. I may perhaps take an example to which I have already referred elsewhere†. The rabbit, the brown rat, the wood mouse, the weasel, and the stoat are numerous and widespread in Great Britain. The wild cat, the pine-marten, the pole-cat, the red-deer, and the yellow-necked mouse are few and locally distributed in Great Britain. Now if we make a few random selections of areas in Great Britain, 20 square miles in extent, the chance of including in one such selection the rabbit, brown rat, wood

* See J. C. Willis, "The Present Position of Darwinism" in *Nature*, 2 Dec. 1922. Also: "Is the Theory of Natural Selection Adequate" in *Nineteenth Century*, Oct. 1922.

† See *Science Progress*, Jan. 1923, p. 476.

mouse, weasel, and stoat is large; the chance of including the wild cat, pine-marten, pole-cat, red-deer, and yellow-necked mouse is small.

And the same mechanical law will have application to the distribution of all living organisms (as also to non-living objects), although of course it may be nullified in special cases by climatic or biological forces, or by the mere fact that in a minority of cases improbable contingencies happen. Consider the species found in a given part of an insular area. Let the whole area be A , and the given part E . And let the species be of varying ranges and distributed fortuitously. Then all the species that are universally distributed in A must occur in the part E . But those that are not universal in A may be missing from E , and if the relative size of E be small, a high percentage of really local species will probably not be found in the fractional area. Thus the average range in A of the species which occur in E will probably be greater than the average range of all the species occurring in A . And we can deduce the general proposition that the average range of the species in any given part of a given area will tend to be greater than the average range of all the species occurring in the whole area. And we can further state that the average range will tend to vary inversely with the size of the geographical fragment considered.

It can further be seen that this principle will apply at once to a section of a country which owing to subsidence has become separated from its mainland; it will apply to continental islands from the moment they become insular. Taking large numbers of species of organisms with varying ranges, or large numbers of inanimate objects fortuitously dispersed, the chances of those species or objects being represented on a severed fragment of a country will vary directly with their ranges in that country. Let us take again the case already mentioned, and suppose that fragments are struck off Great Britain. A moment's consideration will convince anybody that most of the five common mammals would occur on all the fragments, and that on many fragments all five species would occur. But it is equally obvious that a single fragment could hardly include all the five rare species, and that many fragments would miss all five of them. The fauna of the severed fragment is therefore not a true representation of the fauna of the mainland, nor is it a proportionately reduced representation, having the same relative number of widespread and local species. It will tend to be a disproportionately reduced representation, having from the outset a high percentage of the widespread species and a low percentage of the small-range species. And hence the average range on the mainland of species common to the fragment and the mainland would tend to be greater than the average range of all the species occurring on the mainland.

And the case will not be dissimilar if a country be cut into two portions of approximately equal size. Consider an elongated island, A , which by the appearance of a narrow strait becomes converted into two islands, B and C , of equal size. Suppose that large numbers of species, of very various ranges, from universal to very local, are dispersed about A . Now let us consider the average range within B of the species that occur also in C , as compared with the average range of all

the species in *B*. All the species in *B* whose ranges appreciably exceeded half the length of *A*, must occur also in *C*. They will not all appear as long range species within *B* itself, but many of them will do so. Next consider the species of medium range, of a length nearly half *A*. They will occur also in *C*, save only those whose range extends to the extreme end of *B*. These again will not all appear as long range species within *B*, but many of them will do so. Now the great majority of the two groups so far considered will occur in *C* as well as in *B*; but these two groups include all the species appearing as long range species in *B*, although they include also some that are of short range in *B*. Thirdly, let us consider the very short range species occurring in *B*. We have seen that in the case of the medium range species, only the special circumstance of extending to the extreme point of *B* would enable them to avoid occurring in *C*; but with this third lot of species, ranging perhaps in many cases only one-twentieth of *A*, the case is reversed, and they cannot occur in *C*, unless they happened in the first instance to be ranged about the equator of *A*. Thus, most of this third group, consisting of short range species in *B* will not appear in *C*. An inspection of the probabilities in this case therefore brings us to the same conclusion. A high percentage of the long range species of *B* will reach *C*; whilst of the short range species of *B* a smaller percentage will extend to *C*. Or in other words, the average range in *B* of those species which do extend to *C* will tend to be greater than the average range of all the species of *B*. I would point out again that one is dealing here with probabilities, not with a certainty applicable to every case; moreover the probability is itself dependent upon the assumption of very various ranges among the species originally dispersed about *A*. Given unvarying ranges of less than half *A*, and the exact reverse of my proposition would be true; and a very low degree of variation might also produce that reverse result.

There is another point, of a somewhat different character, which is perhaps worth mentioning, although it involves problems which are less simple and obvious than the foregoing. We have seen that the excess of the average range of the species of a given part of a country, over the average range of all the species of a country, will tend to vary inversely as the size of the fractional terrain. The smaller the fragment the higher will the average range of its species (over the whole country) tend to be. But if now we consider the problem as it affects organisms only, not mere inanimate objects, and in a dynamic not merely a static sense—though still on purely mechanical principles—I think that we shall see that we must distinguish between fragments of equal size, according to their position in the whole territory. Consider once more an elongated island running north and south; and consider a point *S* at its northern extremity and a point *T* at its centre. Now *S* and *T* are equally well placed for receiving organisms whose centres of dispersal are in the northern half of the island, each point being at an extremity of the northern half. But *T* is much more favourably placed than *S* for receiving organisms whose centres of dispersal are in the southern half; many short range southern species can reach *T*; only long range southern species can reach *S*. Hence, assuming once more random fortuitous distribution of species of varying

ranges, the average range of the species occurring at T will tend to be lower than the average range of the species occurring at S . A point at the centre of a country will tend to have the best chance of receiving low-range species. Here again, however, we have a proposition which will not necessarily hold good in every case, even on purely mechanical principles; because at an extremity of a country the existence of the periphery will naturally cut short some potential ranges.

I have set out these few elementary considerations because they seem to me to be germane to a statistical study of the distribution of organisms. I think that such statistical investigations may yield interesting results in more than one direction. But just as it would be fruitless to attempt to study flight without considering the law of gravitation and the mechanics of air-pressure, so is it necessary, before proceeding to the biological aspects of geographical distribution, to give due weight to those mechanical laws of probability which must apply to the distribution of all objects, whether non-living or living.

STUDIES ON INSECT METAMORPHOSIS—I. PROTHETELY IN MEALWORMS (*TENEBRIOS MOLITOR*) AND OTHER INSECTS. EFFECTS OF DIFFERENT TEMPERATURES

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(With Plate XI.)

INTRODUCTION AND HISTORICAL.

WHILE in the hemimetabolic insects the nymphs at a very early stage of development possess rudiments of almost all the external organs of the adult, e.g. wings, compound eyes, etc., in the holometabolic forms the appearance of these organs is postponed to a definite later stage—the pupal instar. Certain cases, however, are on record in which the larvae of holometabolic insects also showed some imaginal and pupal organs, a phenomenon for which Kolbe ('03) proposed the term "Prothetely," implying that the organs appear prematurely, due to an accelerated development.

The phenomenon was first definitely pointed out by Heymons ('96), who observed some full-grown mealworms, which had small wing-like outgrowths "echte Flügelanlagen," on the meso- and meta-thoracic regions. The first communication on the subject was, however, made as long ago as 1813 by Majoli (see Hagen, '72) who observed that some silkworms, after the fourth moult, developed directly into moths without spinning a cocoon or passing through a pupal stage. Then Jones ('83) observed a larva of *Melanippe montanata* which possessed antennae and legs of an imago. Since the year 1903, numerous cases of prothetely have been reported: by Kolbe in *Dendrolimus pini*, Riley ('08) in *Dendroides canadensis*, Peyerimhoff ('11) in *Malthodes*-larva, Trägårdh ('12) in *Cantharis*-larva, Kemner ('14) in *Telomatophilus typhae*, Lindner ('15) in *Lymantria dispar*, etc. Thus the phenomenon is now recognised to be of a fairly common occurrence among insects, especially in the Coleoptera and Lepidoptera.

In almost all the above mentioned cases, the changes were observed only in a few specimens, mostly in a single one, and there is almost no detailed record of the previous history or the final fate of the individuals. In spite of the fragmentary data, however, several theories have been propounded to explain the curious phenomenon:

According to Bauer ('04, p. 145) an external mechanical injury in the region of the imaginal discs might bring about an accelerated development of the discs,

and he suggested that some such external influence was responsible for the external development of the wing buds of *Tenebrio molitor*. As pointed out by Heymons, it is very difficult to imagine that a number of individuals can be affected by such an influence at the same time, at the same place, and with an exactly similar degree of intensity as to produce symmetrical wing rudiments, as is the case in the specimens. Moreover, I have actually tried to injure the normal larvae of different stages, as suggested by Bauer, without producing any change whatever.

Equally unconvincing is the theory of Schindler ('02) who believed that external wing development in *Tenebrio* is a consequence of the capacity for regeneration, a characteristic, according to him, possessed by all insect larvae having a heavily chitinised exo-skeleton. To refute this view it is only necessary to recall the above mentioned numerous cases of prothetely in Lepidoptera, where the caterpillars are usually soft bodied, and to remember that there are some insect larvae, e.g. wireworms, which have coats more heavily chitinised than *Tenebrio*, but in which prothetely has never been observed.

The theories of Dewitz ('02, '05) and Heymons ('07) were hailed as the true explanation of the phenomenon; these authors held that in the insect body during development an oxydase is gradually produced, which is responsible for the metamorphosis of the larva into the pupa, that there is a specific oxydase for every set of organs and that it is due to the production in larger quantities of a particular oxydase that its corresponding organs, wing buds, etc., in prothetelic individuals, develop prematurely. If we analyse this theory, it does not appear to solve the problem better than any of the others already mentioned, as we are not told what particular factor is responsible for the greater or less production of the oxydase; Heymons himself admits this a serious shortcoming. He writes (p. 173): "Welcher Natur freilich die Stoffe sein mögen, die den Reiz zur beschleunigten Entwicklung Ausüben oder welche Bedingungen hierzu notwendig sind, darüber lassen sich gegenwärtig wohl kaum Vermutungen aussprechen."

Some believe that prothetely may be simply a case of reversion (atavism), the holometabolic insects showing the characters of hemimetabolic ancestors; and the occurrence of a distinct "Prenymphoid" stage, intervening between the larva and the pupa, and in structure intermediate between the two, reported in the development of *Lebia scapularis* by Silvestri ('05) is considered to be a case of prothetely under normal conditions (Heymons). *Telmatophilus*, according to Kemner, also showed prothetelic features under natural environmental conditions.

In the summer of 1923 while I was breeding *Tenebrio molitor* in another connection at the instance of Mr Balfour Browne, the occurrence of the phenomenon was observed under conditions which could not be harmonised with any of the theories detailed above, and which suggested that the explanation of prothetely should be sought in a totally different direction, and that the phenomenon was not at all a case of accelerated development (on account of which it is named "Prothetely") but rather of retardation. In order to investigate this suggestion fresh experiments were started, the results of which are embodied in the following pages.

I take this opportunity of expressing my thanks to Mr F. Balfour Browne, M.A.,

the Supervisor of my research, who showed great interest in the work, made valuable suggestions, and arranged for all the facilities necessary for carrying out the experiments.

I would also like to acknowledge the great assistance rendered to me by Mr S. A. Arendsen Hein, of the University of Utrecht (Holland), who willingly supplied me with some valuable material and replied to my numerous queries.

OBSERVATIONS AND EXPERIMENTS.

It was observed that in a culture, which was being kept at 27.5°-28° C., while most of the larvae (about 110) had pupated, some remained (about 25) which, as time progressed, grew more and more inert, ceased active feeding, assumed a dull appearance, and began to show small wing-like outgrowths and other characters usually associated with the prothetelic condition. The larvae so affected, if they moulted at all, did so with great difficulty, sometimes taking over six days to cast off a skin; some had still the remnants of the last moulted skin on their legs and mouthparts; some could be seen carrying more than one moulted skin; and in some cases the skin could be peeled off. A few (4) of these larvae pupated, but the pupae were abnormal in appearance, had their wings very much swollen and spread out instead of having them closely applied to the ventral side of the body. Most of the pupae did not succeed in becoming imagines, though the living imago was visible within the pupal skin which the individual evidently could not shed. A few of the larvae, on being put at a lower temperature, 25° C., became more active and lively.

These facts naturally led one to think that the above changes were obviously not due to an accelerated development, but suggested that there was something, apparently high temperature, which was inhibiting the metamorphosis.

To test this hypothesis, on the 2nd October, 1923, 50 normally developing larvae were put at each of three different temperatures, 23° C., 26.5° C., and 29.5° C., while a similar fourth lot was put in a cold room in which the temperature varied from 5° to 11° C. All the four lots were obtained from the same mother culture which had hatched between the 12th and 22nd February, 1923, and was being reared at 27° C., so the difference in age between any two individuals was not more than ten days. The larvae were of a fairly advanced stage of development, and a few pupae had already been obtained from the mother culture. All the lots throughout the experiment were given the same sort and amount of food, although of course all did not consume the same quantity. Unused food was removed every week. With the exception of the one batch in the cold room, all were kept in incubators and thus were equally exposed to the accumulation of a greater or less amount of stagnant air.

By the 2nd week of January, 1924 (see Table I), while 49 out of 50 at 23° C. had pupated, and 46 at 26.5° C. had done the same, only 17 at 29.5° C. and none in the cold room had changed. Of the remaining larvae, those at 23° C. and at the low temperature did not show any sign of wing pads; of the four left

at 26.5° C. one gave an abnormal pupa, somewhat resembling that often formed by a prothetelic larva, while of the 25 living larvae at 29.5° C. ten possessed small rudiments of wings.

Table I.

Temp.	No. of larvae	No. pupated	No. of larvae remaining		No. of those that showed signs of prothetely	Remarks
			Living	Dead		
29.5° C.	50	17	25	8	10	—
26.5° C.	50	46	2	2	1	No signs in the larva, but the pupa showed slight signs of prothetely
23° C.	50	49	1	Nil	Nil	—
$5^{\circ}-11^{\circ}$ C.	50*	Nil	49	1	Nil	*Kept only till the end of November. Then 15 out of these were removed and put at a higher temperature. (See below.)

Of the 50 larvae that were being kept in the cold room, 15 were transferred to 23° C. in the first week of December, 1923. Within eight weeks, four out of these showed distinct wing rudiments.

Results similar to those detailed in Table I were obtained with another series of four lots, made from a culture which had hatched between the 7th and 25th of December, 1922, and which were kept at the same different temperatures (Table II).

Table II.

Temp.	No. of larvae	No. pupated	No. of larvae remaining		No. of those that showed signs of prothetely
			Living	Dead	
29.5° C.	31	4	20	7	6
26.5° C.	31	10	15	6	Nil
23° C.	31	20	8	3	Nil
$5^{\circ}-11^{\circ}$ C.	31	Nil	29	2	Nil

Evidently there have been two factors responsible for the appearance of the prothetelic characters: (i) the prolongation of the larval life by keeping the individuals at too high or at too low a temperature; (ii) and the direct effect of temperature. Thus under these circumstances, while the metamorphosis of the larvae has been inhibited, there has been a tendency for the appearance of the pupal organs at the proper time, when their fellows are pupae or even beetles. Therefore the appearance of the organs is evidently not premature, as was believed to be by previous investigators.

That a very high temperature does delay the development was verified (Table III) by another series of experiments, in which a number of larvae of the same age were

put at different temperatures and a count was made of the moulted skins in a definite period, viz. four months (October-January) in the particular experiments. It will be noticed from the table that the number of skins shed by the larvae at 29.5° C. was one-half, or even one-third, of that shed by those at 26.5° C. or 23° C. Arendsen Hein ('23) who is breeding mealworms from a genetic point of view also observed that with the increase of temperature the pupation is postponed; he writes (p. 156): "Das optimum der Temperatur liegt für obige Larvenarten zwischen 25° C. und 27.5° C. Durch Erhöhung der Temperatur bis 30° C. steigt die Larvenmortalität und wird die Metamorphose verlangsamt." However, the fact that the abnormal larvae are met with in cultures kept at 27.5° C. shows that the optimum temperature is lower than what he believes.

Table III.

Temp.	No. of larvae	No. of moulted skins	No. of larvae	No. of moulted skins	No. of larvae	No. of moulted skins
29.5° C.	40	50	25	30	15	21
26.5° C.	40	155	25	57	15	36
23° C.	40	150	25	60	15	32
5°-11° C.	40	10	25	6	15	2

Arendsen Hein ('20) mentioned that a few of his larvae had wing-like appendages. In reply to a personal communication he has kindly informed me that the conditions under which the above phenomenon had been observed were as follows: "As a rule such larvae appear only in cultures which are about 10-15 months old; in fact, the older the larvae are, the greater the chance of meeting larvae with rudimentary wings." This strongly supports the view that only those larvae show these characters whose larval life has been somehow prolonged or whose pupation has been inhibited.

The above experiments suggest a new and an entirely different line of research: How does high temperature retard the metamorphosis? Does it affect the "agencies" which bring about the histolysis of the larval tissues or does it affect those which are responsible for the histogenesis of the pupal organs? At present there is no unanimous opinion as to the nature of these "agencies." An extensive examination of the histology of the normal individuals and the prothetelic larvae ought to throw some light on the above problems. I have undertaken such a study, but this subject must be reserved for a separate communication.

CHARACTERS OF THE AFFECTED LARVAE.

The most prominent feature, as already mentioned, is the presence of wing-like out-growths. If the larva is sectioned, the above out-growths appear as distinct diverticula of the hypodermis (Fig. 3a, w.). These diverticula are only a part of the future wings, the rest of these lying within the body cavity as large invaginated

pockets (Figs. 3b-3d, *W.*), the openings of the pockets being near the bases of the evaginated portions. It is interesting to point out in this connection that the several investigators whose theories were reviewed on p. 140 assumed that the wings in the abnormal larvae represented rudiments of the whole wings of the future pupa. Moreover, I must record that the wing-like out-growths are not present in the last instar larvae only but are found in earlier stages as well.

In addition to the presence of wing rudiments there are several other structural characters in which the affected larvae are intermediate between the normal larva and the pupa. For instance, the antennae, which are three-segmented in a normal larva and eleven-segmented in a pupa, are composed of five segments (compare Figs. 1d and 1e). Also, some individuals have rudiments of the compound eyes (Fig. 1, *E.*), which are visible through the transparent skin. In size, also, the prothetelic larvae approach pupae in being shorter and broader than the normal individuals (compare Figs. 2a and 2b). The rudiments of a penis and its appendages, which ordinarily assume prominence in the pupal stage, are fairly developed in some of these larvae (Fig. 2a). Then, the testes, very small and almost unrecognisable from the surrounding fat tissue in the normal larvae, are very prominent in the affected individuals (cf. Figs. 1, 1a, and 1b, *tes.*); in size they approach those of the pupa, but unlike the latter they are more or less fused with each other. The testes were sectioned; they did not show any spermatozoa. The ovaries did not show any advance.

There is no doubt that the larvae cannot moult freely—and consequently sometimes assume a very interesting form, *e.g.* an individual sheds its last larval skin from the abdomen, but is unable to do so from its head and thoracic region; it then resembles neither a pupa nor a larva as it has the head and thorax of the former and the abdomen of the latter. The wings of both the pupae and the beetles formed from the affected larvae contain a large amount of thick fluid.

GENERAL CONCLUSIONS.

The appearance of imaginal organs in the *Tenebrio molitor* larvae which happens only in those individuals whose pupation is overdue, and which obviously is not a result of an accelerated development, can no longer be regarded as an example of prothetely in the sense in which it has hitherto been understood. Examining other cases on record, for instance, Majoli's silkworms, which under the influence of high temperature developed directly into "moths" without pupation or spinning a cocoon, it seems that their condition was almost the same as that of the abnormal *Tenebrio* larvae. The statement that the caterpillars after the fourth moult developed into moths cannot be correct on a morphological basis, as there must be, before the moth-stage can be said to be reached, two more moults, one before the pupation, the other before the emerging of the moth; therefore Majoli's moths were, theoretically, caterpillars, with, however, large wings, larger than those in the affected mealworms. Moreover, in structure, also (as is clear from their anatomical description reproduced by Hagen ('72)) they were caterpillars rather than moths,

except for the presence of wings. Hagen writes: "Dieser Schmetterling unterscheidet sich von dem Seidenspinner durch folgende Merkmale. Er hat eine kleinen Kopf, zwei schwarze gegitterte Augen, den Thorax, wie wenn er der dritte Ring hinter dem Kopfe bei der Raupe wäre und den Körper der Raupe selbst, wie er zur Zeit der vierten Hautung ist, mit ebenso viel Segmenten, wie der Raupen Körper; die Vorderflügel etwas lang und verschmäler, die Hinterflügel kürzer und schmäler; die Fühlhorner etwas grau, im vergleich mit denen des wahren Seidenspinner."

Majoli's specimens, therefore, differed from the abnormal mealworms only in degree and not in kind. Moreover, as they were also being reared at a very high temperature, it is highly probable that they were also suffering from inhibited metamorphosis.

Kolbe's prothetelic larva of *Dendrolimus pini* was one of the six larvae sent by Winneguth to him on 25th June, 1902. These caterpillars, according to Winneguth, belonged to a second generation; the first generation had hatched in January, 1902, the first imago was observed on the 30th May, the rest following soon after. Now, the abnormal larvae, which are described as of the next generation, were discovered about the 25th June, and one of these larvae was reported to have pupated and even formed a deformed imago by this time. It seems well-nigh impossible that they could have reached the stage of development attributed to them in a period of less than two weeks, which would have been their age if they had been the offspring even of the first imago which emerged about the 30th May, allowing about 10 or 12 days for the laying and hatching of the eggs. Therefore it seems highly probable that the six larvae were left over from the first generation and that they had not been able to pupate and become adults like their fellows. If that is the case then this is also an example of retarded metamorphosis as is *Tenebrio* and the silkworm of Majoli. The fact that the moth that was formed by one of these larvae was abnormal reminds us of the deformed *Tenebrio* beetles obtained from the affected larvae.

In the absence of any recorded facts about other reported cases, in almost all of which only one abnormal specimen was found, I am not in a position to discuss their nature.

HYSTEROTELY.

While Kolbe gave the name "prothetelie" to the phenomenon of the presence of imaginal organs in larvae, Schulz ('22) proposed the term "Hysterotelie" for the opposite phenomenon of the presence of larval organs in the imago, to cover several cases on record in which butterflies or moths have been found to possess caterpillar-heads. The earliest case is that recorded by Müller ('44) who found such a butterfly and described it as a new species. He was, however, corrected by Werneburg ('64) who showed that Müller's new species was merely a specimen of *Lymantria monacha* with an abnormal head. Hagen ('72, pp. 380-402) gives several similar cases in which a butterfly or moth possessed a caterpillar-head.

In almost all these cases, except that reported by Schulz, it was found that inside the caterpillar-head there was a pupal-head, and often inside the latter the crumpled

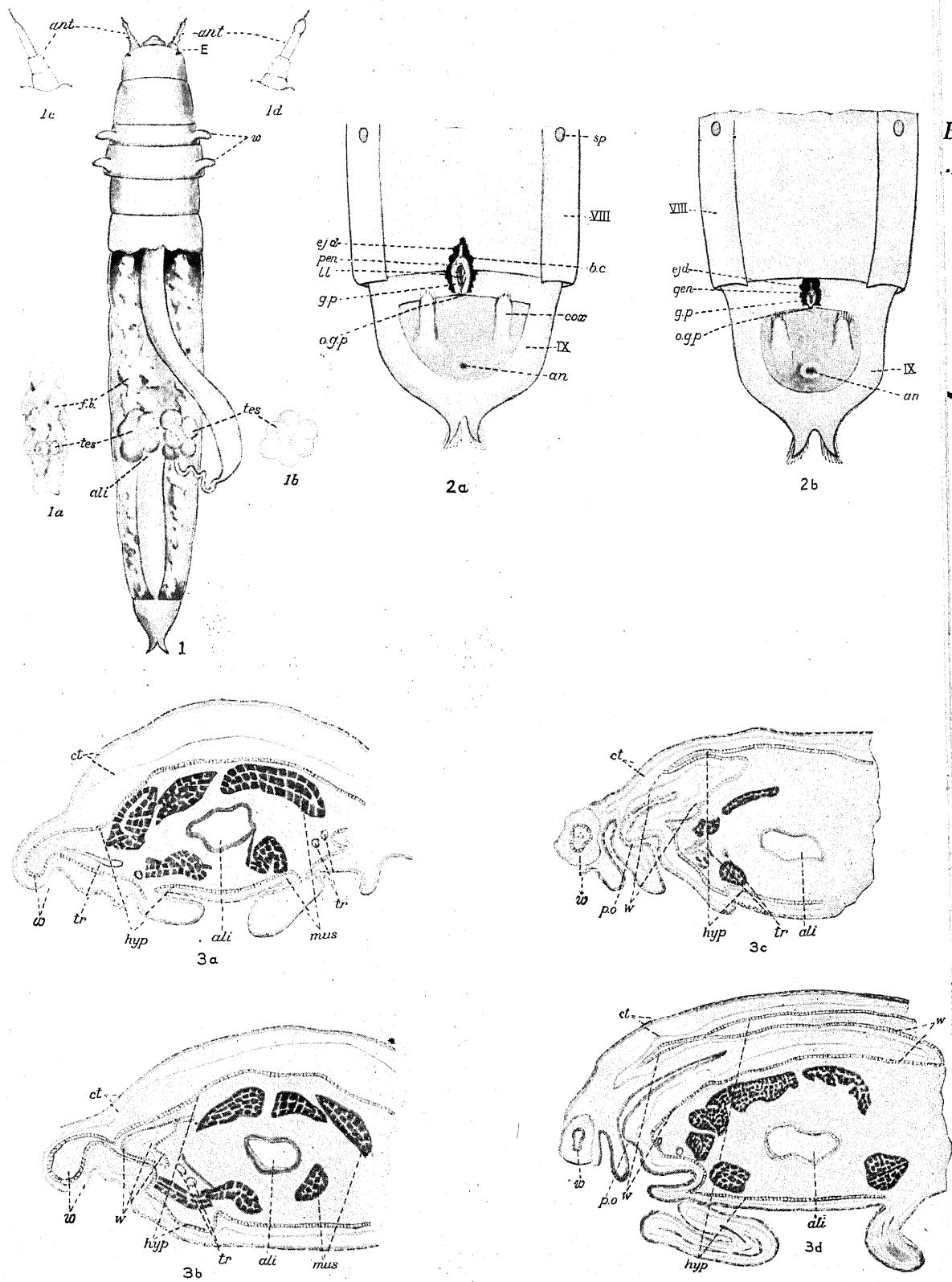
imago-head itself could be made out. Therefore in these cases it seems that while the individuals had been able to cast off the larval skin from the rest of the body, they were unable to do so in the head region. So that here again we seem to have the same condition as in *Tenebrio*. On page 144 I have described a specimen in which the head and thorax were those of a larva, and the abdomen that of the pupa, a condition approaching that of the hysterotelic individuals.

Therefore almost all the known cases of both Prothetely and Hysterotely are really cases of the same phenomenon in which the pupation or the metamorphosis of the larvae is inhibited, *i.e.* they fall within the domain of Nooteny. Hence, while the term "Hysterotely" is only superfluous, "Prothetely" is both superfluous and bad.

Just before sending this paper to the Press, I received a copy of *Prothetelie bei Coleopteren-larven*, by H. v. Lengerken, which has recently been published ('24). This author concludes that before we can definitely decide the nature of "prothetelie" several questions must be answered, *e.g.* Do the prothetelic larvae pupate? Are the affected larvae those of the last moult or are the wing rudiments present in the younger larvae as well? It is hoped that the observations here described throw light on these questions.

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EXPLANATION OF FIGURES OF PLATE XI.

FIG. 1. A prothetelic larva, dissected to show testes, etc. *ali.* alimentary canal; *ant.* antennae; *E.* compound eye; *f.b.* fat body; *tes.* testes; *w.* wing rudiments.

FIG. 1 *a*. Testes of a normal fully developed larva.

FIG. 1 *b*. Testes of a normal fully developed pupa.

FIG. 1 *c*. Antenna of a normal fully developed larva.

FIG. 1 *d*. Antenna of a prothetelic larva.

FIG. 2 *a*. Ventral view of the posterior abdominal segments of a prothetelic larva, dissected to show the developing penis and its appendages, etc. *an.* anus; *b.c.* body cavity; *cox.* coxites; *ej.d.* ejaculatory duct; *g.p.* genital pocket; *l.l.* lateral lobes, the appendages of the penis; *o.g.p.* opening of the genital pocket; *pen.* penis; *sp.* spiracle; *VIII.* eighth abdominal segment; *IX.* ninth abdominal segment.

FIG. 2 *b*. Ventral view of the posterior abdominal segments of a normal larva; magnified to the same scale as Fig. 2 *a*. *gen.* knob-like rudiments of the future external genitalia, both penis and its appendages, which are yet indistinguishable from each other.

FIGS. 3 *a-3 d*. Transverse sections through the different regions of the thorax of a prothetelic larva. *ct.* chitinous cuticle; *hyp.* hypodermis; *mus.* muscles; *tr.* trachea; *w.* evaginated portions of the future wings; *W.* invaginated future wings.

OOGENESIS OF *LITHOBIOUS FORFICATUS*

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(With Plates XII, XIII.)

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I. INTRODUCTION.

IN 1902 Carl Tonniges (18) investigated the oogenesis of *Lithobius forficatus*, but he confined himself exclusively to the origin of the oocytes from the indifferent cells of the germinal epithelium. To the best of my knowledge, no other account of oogenesis in *Lithobius* has ever been given.

In the present paper an attempt has been made to work out in detail the remarkable deeply basophil extrusions given out by the amphinucleolus of the oocyte nucleus into the cytoplasm and their possible function; the structure, fragmentation and the ultimate transformation of the Golgi apparatus into the fatty yolk which is deeply blackened even by chrome-osmium and which is distinct from the true vitelline yolk; and the mitochondria. A study of the centrifuged egg has also been made and the nature of the various cell inclusions thus conclusively settled. At the end of the paper the reactions of the cell inclusions to the various fixatives and stains used are given in a tabular form.

I have to thank Mr J. Gray, M.A., under whose supervision this work has been carried out, for the keen interest he has taken in this work and also for reading and correcting the manuscript of this paper.

II. TECHNIQUE.

The presence of very large and brittle yolk discs in the more highly developed oocytes is a serious impediment in the way of cutting thin sections even by the paraffin-celloidin method. Sections as thick as 16μ , therefore, had to be cut and these proved highly satisfactory. For the study of the Golgi apparatus and the mitochondria, however, fully developed oocytes were removed from the ovary and sections as thin as 6μ were thus easily obtained.

The following fixatives and stains were used:

- (1) Bouin's fluid followed by iron haematoxylin. Fuchsin and eosin were used as counter-stains in many cases.
- (2) Altmann's fluid followed by Altmann's acid fuchsin and picric acid.
- (3) Champy-Kull's fluid followed by Altmann's acid fuchsin, thionin and aurantia.
- (4) Flemming-without-acetic followed by iron haematoxylin.
- (5) Champy-Kull's fluid followed by Benda's alizarin and crystal violet.
- (6) Prolonged osmication method of Mann-Kopsch followed sometimes by Altmann's acid fuchsin.
- (7) Da Fano's silver nitrate precipitation method followed by safranin.

For the Golgi apparatus the Mann-Kopsch method proved much more satisfactory than Da Fano's method.

III. NUCLEOLUS AND ITS DERIVATIVES.

Fig. 1 shows an oocyte in the resting condition. The nucleolus stains deeply and uniformly with haematoxylin. The amount of basiphil material in it preponderates at this stage, and it, therefore, does not stain with acid stains. A little later (Fig. 2) the nucleolus enlarges and becomes slightly irregular in outline; it still stains deeply and uniformly with basic stains. A number of deeply basiphil bodies are plastered on the outside of the nuclear membrane and one such body lies near the nucleolus. These basiphil bodies originate from the nucleolus.

As growth of the oocyte and its nucleus proceeds, the nucleolus shows signs of intense activity. It increases considerably in size and clearly becomes an amphineucleolus (Fig. 3). It shows a number of deeply basiphil bodies embedded in an acidophil ground substance. Some of these bodies are circular, while others are irregular in outline (Figs. 3, 4 and 5). As a rule the circular bodies remain within the nucleus and form secondary nucleoli, whereas the irregular masses are first plastered round the nuclear membrane, they are later detached from it and thus come to lie in the cytoplasm (Fig. 5). These masses will henceforth be called nucleolar extrusions.

The secondary nucleoli, when first formed, are deeply basiphil. In Fig. 3 a large number of deeply basiphil secondary nucleoli are present, but at S.N.¹ are two secondary nucleoli each of which shows a deeply basiphil body embedded in an acidophil ground substance. Gradually, however, all the secondary nucleoli become completely acidophil (Figs. 4, 5 and 7). Lastly when the nucleus takes its

position just below the chorion in a patch of protoplasm free from yolk, the secondary nucleoli all disappear (Fig. 8).

The nucleolar extrusions are much larger than the secondary nucleoli. They are so numerous that almost any section will demonstrate them. Indeed, it is remarkable that Tonniges⁽¹⁸⁾ omitted to mention them. When plastered round the nuclear membrane they may bud off smaller pieces into the cytoplasm (Fig. 3, *N.E.*¹). Sooner or later all the nucleolar extrusions are detached from the nuclear membrane and lie in the cytoplasm.

When first formed the nucleolar extrusions stain deeply and uniformly with basic dyes like the secondary nucleoli. Soon they show differentiation in their substance. Fig. 12 is a Champy-Kull preparation. At *N.E.* two nucleolar extrusions are shown. The unshaded portion is the acidophil ground substance in which are a number of vacuoles. This stains with acid fuchsin. In the centre is a basophil mass staining green with thionin. Similarly in Fig. 6 (which is a Bouin preparation) is shown a nucleolar extrusion lying amidst a number of yolk granules. There is one large irregular basophil body with a number of smaller ones embedded in an acidophil ground substance. The nucleolar extrusion itself is lying in a vacuole across which run a number of fine strands. The vacuole seems to be surrounded by a membrane. Indeed the whole structure looks like a secondary nucleus. It must be mentioned, however, that vacuoles are present round a few nucleolar extrusions only and the strands are present round still fewer vacuoles. It is, therefore, likely that vacuoles are artificially formed by the sudden coagulation of the nucleolar extrusion. Ultimately the nucleolar extrusions become completely acidophil and later disappear; exactly how cannot be determined.

The amphinucleolus, after the process of nucleolar extrusions has ceased, becomes completely acidophil and prominent vacuoles appear in it (Figs. 7 and 8).

The connection between nucleolar extrusions and yolk formation if any, will be discussed later. Here it need only be said that the formation of nucleolar extrusions precedes yolk formation, although a few extrusions may exist side by side with yolk (Fig. 6).

IV. CHROMOSOMES.

Fig. 1 shows a resting oocyte. No chromatin network can be clearly made out in the nucleus. A little later, however, the chromosomes appear and are present in pairs (Fig. 2). This is the zygotene stage. Henceforth the chromosomes remain in pairs, although they become achromatic and cannot, therefore, all be made out (Figs. 3, 4, 7). When the nucleus moves just below the chorion in a patch of protoplasm free from yolk, the chromosomes disappear altogether (Fig. 8). Fig. 8 shows a portion of the most highly developed oocyte which can be obtained from the ovary. It is very likely, therefore, that the chromosomes will become chromatic some time after the oocyte leaves the ovary. As the present work has been done from the point of view of cell inclusions, the study of polar bodies has not been attempted. It may, however, be added that such study will be extremely difficult, if not impossible, on account of the presence of the huge yolk discs.

V. THE GOLGI APPARATUS, THE FATTY YOLK AND THE VITELLINE YOLK.

In a young oocyte (Fig. 9) the Golgi apparatus consists of small rods collected in one patch lying on one side of the nucleus. No archoplasm is associated with the rods. Fig. 9 is a Mann-Kopsch preparation. With Da Fano's silver nitrate precipitation method the individual rods are difficult to make out and the whole apparatus appears as a very dark structure containing small shining spots. This appearance is, of course, due to the extreme reduction of the silver.

As the oocyte grows, the apparatus begins to fragment profusely (Fig. 10). At certain places the rods are separate from each other, while at others they lie very close together and thus small network-like structures are formed.

A close study of Mann-Kopsch preparations shows that the Golgi rods undergo fatty degeneration and give rise to fatty yolk as distinct from true vitelline yolk. Fig. 11 is an unstained Mann-Kopsch preparation. At *G.A.*¹ is the Golgi apparatus. There are a number of round bodies at *F.y.* lying near, as well as in, the apparatus itself. Indeed, these bodies can be clearly seen originating from the Golgi apparatus. Again, when the fatty yolk is increasing, there is a corresponding decrease in the Golgi rods till at a late stage no Golgi rods can be detected.

The fatty yolk granules when first formed are small, but later they grow in size. That they contain free unsaturated fat is made clear by the fact that they are intensely blackened by chrome-osmium alone, whereas Golgi rods proper are blackened by prolonged osmication only (Mann-Kopsch method). Fig. 12 is a Champy-Kull preparation and the solid granules at *F.y.* are the fatty yolk. They appear quite black in unstained sections. The Golgi rods which are not yet changed into the fatty yolk, of course, do not appear in this preparation. Further, the fatty yolk does not appear in Bouin's preparations, whereas true vitelline yolk and nucleolar extrusions are preserved (Figs. 6, 7 and 8). Again true vitelline yolk is very slightly, if at all, blackened by osmic acid and can be decolorised in turpentine in about one minute, whereas the fatty yolk is decolorised after at least fifteen minutes. Thus a distinction can be easily made between the fatty yolk and the vitelline yolk. Lastly the vitelline yolk appears a considerable time after the fatty yolk has appeared.

That the Golgi rods give rise to fatty yolk can be proved conclusively by studying the centrifuged eggs. This evidence, however, is reserved for a separate section.

Fig. 13 is a Da Fano preparation. The Golgi rods exist separately throughout the oocyte and also collectively in the form of very close networks. The fatty yolk is, like the mitochondria, stained golden in untoned sections and cannot thus be made out with certainty. In toned sections both the mitochondria and the fatty yolk appear grey.

VI. MITOCHONDRIA.

The mitochondria are not of any special interest. They exist as minute spheres uniformly distributed throughout the oocyte (Fig. 12). They are stained pink with Altmann's acid fuchsin, violet with Benda's crystal violet and black with iron

haematoxylin. In Mann-Kopsch unstained sections they appear yellowish. In very young oocytes they do not appear, but when a little later they do make their appearance, they do so suddenly. For example, in Da Fano untoned preparations the cytoplasm of very young oocytes stains uniformly yellow and no mitochondria can be made out, but a little later they suddenly appear and are of a beautiful golden colour. This would suggest a *de novo* origin of the mitochondria.

VII. CENTRIFUGED EGGS.

The study of centrifuged eggs has proved very useful in *Lithobius*. Live animals are centrifuged for about half an hour. The head of the animal is cut and the ovary is dissected out in normal salt solution as soon as possible. It is then immediately thrown into the fixative. Various fixatives were used and the following results obtained.

When a centrifuged ovary fixed by Mann-Kopsch method is mounted unstained, a very dark band appears at one pole of the oocyte (Figs. 14 and 15, *F.y.*). This band is blackened so intensely and uniformly by osmic acid that no structure can be made out in it. If, however, the slide is kept in turpentine for about fifteen minutes, the dark band is decolorised and in it definite spheres appear. This is the fatty yolk which arises from the Golgi rods. At the opposite pole (Figs. 14 and 15, *V.y.*) there are the true vitelline yolk granules which are not stained black by osmic acid but appear yellowish brown. The very small amount of blackening which they may undergo is removed in about one minute by turpentine. The middle area is full of mitochondria (Figs. 14 and 15, *M.*) which appear yellowish, and this area contains the nucleus also. The nucleus does not lie in the centre of the middle area but nearer the band of the fatty yolk. A few Golgi rods which are not yet transformed into the fatty yolk also lie scattered in the middle area (Figs. 14 and 15, *G.A.*). Fig. 15 shows a much older oocyte than Fig. 14, and it will be noticed that the unchanged Golgi rods in the former are much fewer than in the latter, clearly showing the decrease in Golgi rods and the corresponding increase in the fatty yolk.

The statement that the Golgi rods are transformed by a fatty degeneration into the fatty yolk is, therefore, supported both by direct and indirect evidence. Direct evidence is illustrated by Fig. 11 where at *G.A.*¹ the fatty yolk is actually arising from the Golgi apparatus, and indirect evidence is obtained by the decrease in Golgi rods and the corresponding increase in the fatty yolk (Figs. 14 and 15).

When centrifuged eggs are fixed with Da Fano's silver precipitation method most beautiful preparations result. The true vitelline yolk is thrown down and is somewhat grey, the fatty yolk at the opposite pole appears golden brown and the central area containing the mitochondria appears golden. The few unchanged Golgi rods are blackened and lie in the central area, and so does the nucleus. The fatty yolk is not so well preserved with Da Fano as with Mann-Kopsch.

With Champy-Kull fixation all inclusions appear except the unchanged Golgi rods, whereas with Bouin's fluid the true vitelline yolk alone is preserved.

It must be said at the end of this section that, had it not been for the study of the centrifuged eggs, it would have been difficult to establish satisfactorily the fact that the fatty yolk arises from the Golgi rods.

VIII. DISCUSSION.

(a) *Nucleolar extrusions and yolk formation.*

The presence of chromatoid bodies in the cytoplasm of the oocytes in Metazoa has been observed in a very large number of cases, particularly among the insects [e.g. Hymenoptera (Gatenby (7), Hegner (9), Blochman (2), Korschelt (11), Loyez (12), Buchner (3)); Diptera, Lepidoptera, Hemiptera and Coleoptera (Stuhlmann (17)); Orthoptera (Buchner (4))]. In groups other than the Insect group they have been described in sponges by Dendy (6); in the Polychaete *Aricia foetida* and in *Strongylocentrotus* by Schaxel (15, 16); in *Antedon* by Chubb (5); in Copepod oocyte by Moroff (13); in Paludina by Popoff (14); and in the amphibian *Proteus anguineus* by Jorgenson (10).

On examining closely this huge mass of literature one finds that in a comparatively few cases only, the origin of these chromatoid bodies in the oocyte cytoplasm from the nucleus can be said to have been satisfactorily established. Indeed, their origin from the nucleus has been denied in many cases, e.g. by Beckwith (1), who denies such origin in the eggs of Hydrozoa in which Schaxel had described nuclear extrusions. In still fewer cases their origin from the nucleolus has been established, e.g. in *Antedon* by Chubb (5) and quite recently by Paul Buchner (3) in *Rhysa*. Nevertheless careful research by recent cytological methods has established in many cases that these chromatoid bodies in the oocytes react to chromatin stains in a way similar to chromatin itself, and also that they originate from the nucleus or the nucleolus, e.g. in *Saccocirrus* (Gatenby (8)).

In *Lithobius* it can be seen very clearly that the nucleolus is first deeply basophil; later it becomes amphophil with an acidophil ground substance embedded in which are deeply basophil circular bodies and comparatively bigger and irregular masses which are discharged into the nucleus (Fig. 3). The circular bodies as a rule remain within the nucleus and become secondary nucleoli, while the larger irregular masses are at first plastered round the nuclear membrane and here they may bud off small pieces in the cytoplasm. Later they are detached from the nuclear membrane and lie in the cytoplasm. At first they are deeply basophil, then they become amphophil and ultimately completely acidophil. In their reaction to stains these nucleolar extrusions behave exactly similar to the nucleolus itself.

There is no direct evidence to show that these nucleolar extrusions are actually transformed into yolk. All that can be said with certainty is that they precede yolk formation, although a few may exist side by side with yolk. It is quite possible, however, that when the nucleolar extrusions disappear, their substance may contribute towards the nutrition of the oocyte. Indeed, direct evidence for this physiological problem is very difficult, if not impossible, to obtain by the usual methods of cytological technique. It is also possible that the nucleolar extrusions

may exert a formative influence in the elaboration of yolk, much in the same way as the nucleus is supposed to exert its morphogenetic influence.

No useful purpose will be served by labouring this point or by discussing other theories, mostly unsupported by direct evidence, concerning the possible functions of nuclear extrusions. Nevertheless I must emphasise the following two facts which, to my mind, clearly show, although perhaps not quite conclusively, that these extrusions are concerned in the elaboration of yolk.

(1) To the best of my knowledge there is no clearly established case of nuclear extrusions in the metazoan oocytes in which yolk is not ultimately developed. Again nuclear extrusions almost always precede yolk formation.

(2) Although it will shortly form the subject matter of a separate paper, I can illustrate the present point by several genera of scorpions. It is well known that in some forms of scorpions, e.g. *Palamnoeus*, there is no yolk in the oocyte, whereas in the other forms, e.g. *Buthus* and *Euscorpius*, the oocytes have a considerable amount of yolk. Now I have clearly established that in the oogenesis of *Palamnoeus fulvipes madraspatensis* the nucleolus does not show any activity and remains small till it disappears when the bivalents condense. On the other hand in *Buthus judaicus* and in *Euscorpius napoli*, the nucleolus is first small and deeply basiphil; later it increases considerably in size and becomes amphophil with an acidophil ground substance in which are embedded circular deeply basiphil bodies. These bodies are discharged in large numbers into the cytoplasm, develop distinct vacuoles round them and form secondary nuclei which give a characteristic appearance to the cytoplasm at this stage. They all disappear before the appearance of yolk.

These facts irresistably lead, I think, to the conclusion that nucleolar or nuclear extrusions are concerned in the elaboration of food materials; exactly how cannot be ascertained.

(b) *Golgi apparatus and fatty yolk.*

Both direct and indirect evidence has been adduced in support of the statement that in the oocyte of *Lithobius* the Golgi rods are transformed by fatty degeneration into fatty yolk.

The only other case, so far as I know, in which the Golgi rods are changed into a different material in oogenesis is that of *Saccocirrus* described by Gatenby (8). Speaking of centrifuged eggs of *Saccocirrus* Gatenby says that "the upper cap is formed of delicate granules which, I think, are fatty yolk and probably of the Golgi elements; these granules will go yellowish green after prolonged osmication." In *Lithobius*, however, the fatty yolk is blackened intensely even after chrome-osmium, indicating the presence of a large amount of free unsaturated fat in its constitution.

The Golgi apparatus seems to have clearly, therefore, a nutritive function in the oogenesis of *Lithobius* and *Saccocirrus*. Unfortunately the Golgi rods in oocytes have been worked out in a few cases only. It will, therefore, be of some interest to ascertain their ultimate fate in as many cases as possible.

Inclusions in the Oogenesis of Lithobius.

Method employed	Bouin's picro-formol, iron haematoxylin and fuchsin	Champy-Kull	Flemming-without-acetic and iron haematoxylin	Benda	Da Fano	Mann-Kopsch
Golgi apparatus	Does not appear	Does not appear	Does not appear	Does not appear	Black both in toned and untoned sections.	Black
Fatty yolk	Ditto	Black in unstained sections. Can be decolourised in fifteen minutes	Same as for Champy-Kull	Same as for Champy-Kull	Golden brown in untoned sections. Grey in toned ones	Same as for Champy-Kull
Mitochondria	Do not appear	Pink	Black	Violet	Yellowish in unstained sections. Can be stained pink with Altmann's acid fuchsin	Yellowish in unstained sections. In untoned sections grey
Nucleolar extrusions	Black, or both black and red, or red only according to the relative amounts of basiphil and acidophil materials	Green, or both green and red, or only red	Black of varying degrees	Red	Do not appear but can be stained red with safranin after toning	Yellow in unstained slides
Secondary nucleoli	Ditto	Ditto	Ditto	Ditto	Ditto	Ditto
Vitelline yolk	Yellow in unstained slide. Black in stained ones	Very slightly blackened. Can be decolourised in turpentine in one minute	Same as for Champy-Kull	Same as for Champy-Kull	Grey both in toned and untoned sections	Same as for Champy-Kull

IX. SUMMARY.

1. The nucleolus of the oocyte nucleus is at first basophil, then amphophil and finally completely acidophil showing prominent vacuoles. It discharges into the nucleus deeply basophil circular and irregular masses of a chromatin-like substance. The former remain in the nucleus and form secondary nucleoli which, becoming acidophil, ultimately disappear. The irregular masses, the nucleolar extrusions, are first plastered round the nuclear membrane where they bud off small pieces in the cytoplasm. Later they are detached from the nuclear membrane and lie in the cytoplasm where they become amphophil and then acidophil. As a rule they disappear before the vitelline yolk puts in its appearance.

2. Strong evidence has been adduced by a comparative study of the various genera of scorpions with and without yolk in the oocytes in favour of the view that nucleolar extrusions are probably concerned in yolk formation.

3. The Golgi apparatus in young oocytes consists of small rods collected in a patch on one side of the nucleus. Evidence, both direct and indirect, has been adduced in favour of the view that Golgi rods are transformed into fatty yolk. The Golgi rods, therefore, have a nutritive function in the oocyte of *Lithobius*.

4. The fatty yolk is blackened even by chrome-osmium, indicating the presence of free unsaturated fat in its constitution. The true vitelline yolk appears a considerable time after the fatty yolk and goes yellowish brown in osmic acid.

5. In a centrifuged egg fixed by prolonged osmic acid method the fatty yolk forms a black band at one pole, whereas the vitelline yolk is thrown down at the other pole. The middle area of the egg consists of the mitochondria, the nucleus and the unchanged Golgi rods whose amount is, roughly speaking, inversely proportional to that of the fatty yolk.

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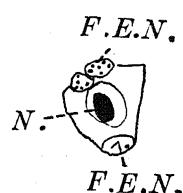


Fig. 1

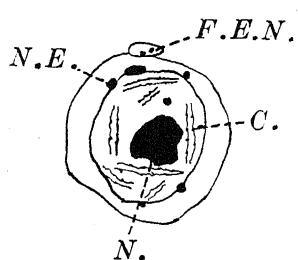


Fig. 2

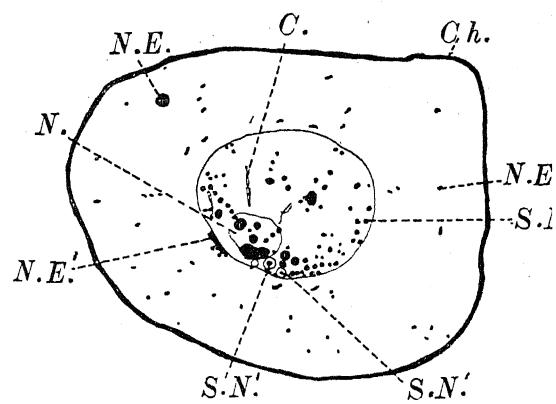


Fig. 3

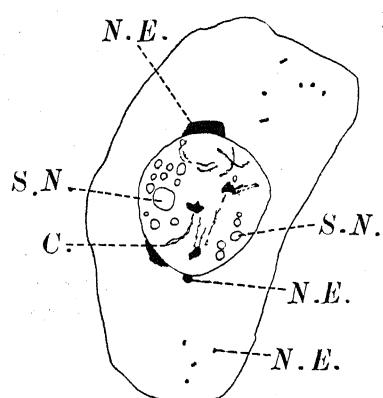


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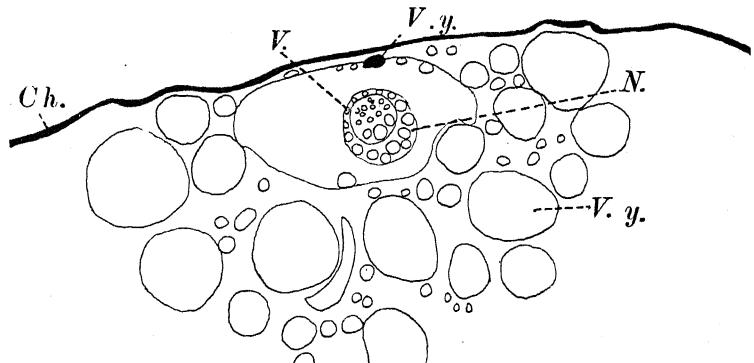


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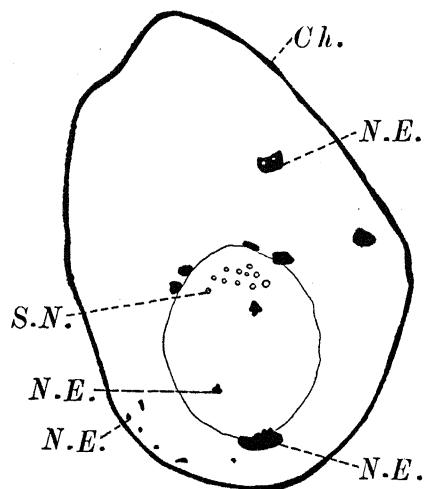


Fig. 5

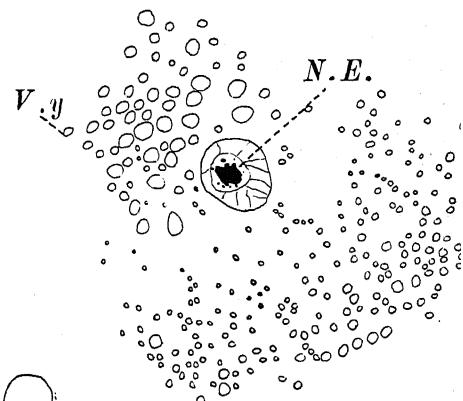


Fig. 6

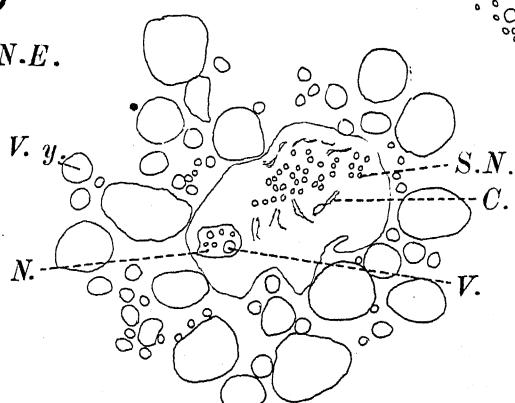


Fig. 7

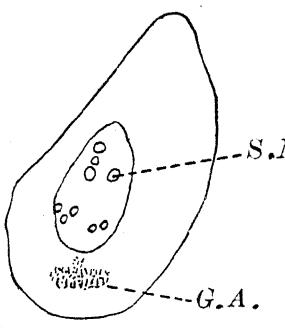


Fig. 9

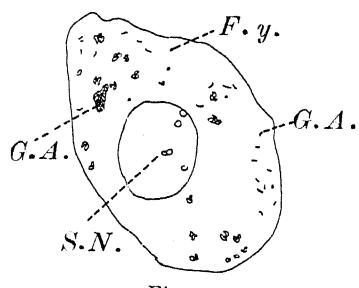


Fig. 10

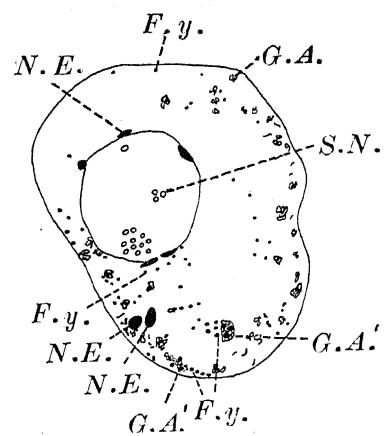


Fig. 11

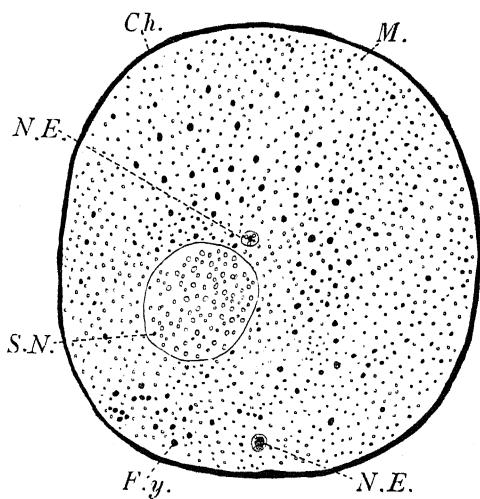


Fig. 12

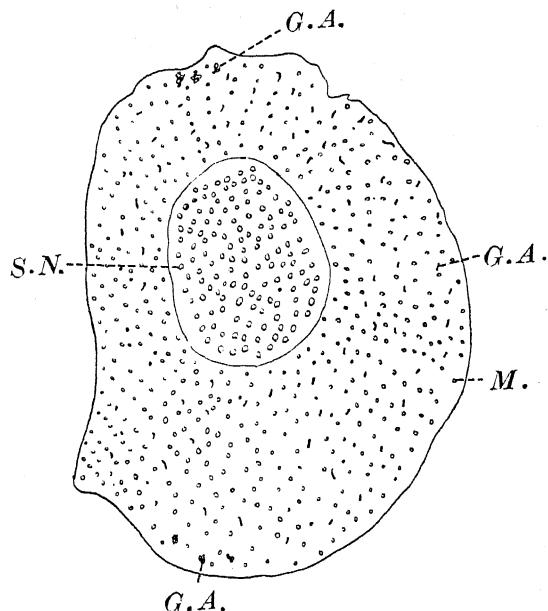


Fig. 13

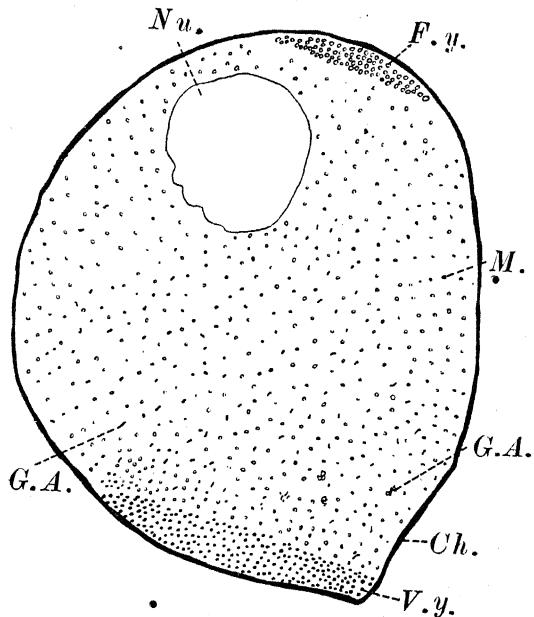


Fig. 14

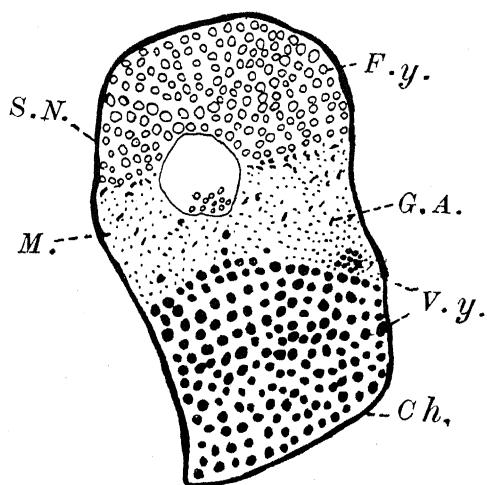


Fig. 15

XI. EXPLANATION OF PLATES XII, XIII.

C. chromosomes; *Ch.* chorion; *F.E.N.* follicular epithelium nucleus; *F.y.* fatty yolk; *G.A.* Golgi apparatus; *G.A.¹* Golgi apparatus forming fatty yolk; *N.* nucleolus; *Nu.* nucleus; *N.E.* nucleolar extrusions; *N.E.¹* nucleolar extrusion budding off smaller pieces; *S.N.* secondary nucleoli; *S.N.¹* secondary nucleoli having both basophil and acidophil materials; *V.* vacuole; *V.y.* vitelline yolk.

FIG. 1. An oocyte with its nucleus in the resting stage. Nucleolus basophil. Bouin. $\times 1680$.

FIG. 2. An oocyte with the chromosomes in the zygotene stage. Nucleolus is still basophil but has become bigger and irregular in outline. Nucleolar extrusions plastered round the nuclear membrane. Bouin. $\times 1680$.

FIG. 3. An oocyte with highly achromatic chromosomes; only three pairs can be made out. Copious discharge of chromatin-like bodies from the nucleolus. Bouin. $\times 488$.

FIGS. 4, 5. Showing huge nucleolar extrusions plastered round the nuclear membrane and also lying in the cytoplasm. Bouin. $\times 488$.

FIG. 6. Showing a nucleolar extrusion in the Amphophil condition lying in a prominent vacuole and surrounded by vitelline yolk. Bouin. $\times 488$.

FIG. 7. Showing the nucleus of an advanced oocyte. Nucleolus has become completely acidophil and prominent vacuoles have appeared in it. Huge yolk discs. Bouin. $\times 488$.

FIG. 8. Showing a portion of the most highly developed ovarian oocyte. Chromosomes have disappeared altogether. Nucleolus with prominent vacuoles. Nucleus lies just below the chorion. Bouin. $\times 488$.

FIG. 9. Showing unfragmented Golgi apparatus. Mann-Kopsch; unstained. $\times 1680$.

FIGS. 10, 11. Showing the fragmentation of the Golgi apparatus and the formation of fatty yolk from the Golgi rods. Mann-Kopsch; unstained. $\times 549$.

FIG. 12. Showing mitochondria, fatty yolk and two nucleolar extrusions in the amphophil condition. Champy-Kull. $\times 366$.

FIG. 13. Showing mitochondria and Golgi rods. Da Fano. $\times 549$.

FIGS. 14, 15. Centrifuged oocytes showing three distinct regions. Note the increase in fatty yolk in Fig. 15 and the corresponding decrease in the Golgi rods. Fig. 15 shows a much older oocyte. Mann-Kopsch. Fig. 14 ($\times 488$); Fig. 15 ($\times 198$).

A MATHEMATICAL THEORY OF NATURAL AND ARTIFICIAL SELECTION. PART II

THE INFLUENCE OF PARTIAL SELF-FERTILISATION, INBREEDING, ASSORTATIVE MATING, AND SELECTIVE FERTILISATION ON THE COMPOSITION OF MENDELIAN POPULATIONS, AND ON NATURAL SELECTION.

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IN the first paper⁽¹⁾ of this series expressions were found for the effect of natural selection of small and constant intensity on Mendelian populations whose generations do not overlap; either during random mating, or when all zygotes are self-fertilised. An intermediate condition as regards mating may arise when there is a tendency to self-fertilisation, to mating between relatives, or to unions between similar but not necessarily related zygotes or gametes. We consider a population whose m th generation consists of $p_m AA : 2q_m Aa : r_m aa$, where A is a completely dominant Mendelian factor, and $p_m + 2q_m + r_m = 1$. When such a population is subjected to any system of mating it falls rapidly or instantly into a new equilibrium. During this process it will be shown that the gametic ratio $u_m = \frac{p_m + q_m}{q_m + r_m}$ is unaltered. When equilibrium is reached under the given mating system we find p, q, r in terms of u .

We now suppose selection to take place at such a rate that $(1 - k)$ recessives survive for every dominant, and so slowly that the population is always very nearly in equilibrium under the mating system. If this condition were not fulfilled we should have to investigate the problem by the method of Lotka⁽²⁾, which in this case presents considerable difficulties. During selection we have

$$u_{n+1} = \frac{p_n + q_n}{q_n + r_n - kr_n},$$
$$\therefore \Delta u_n = \frac{kr_n u_n (1 + u_n)}{1 - kr_n (1 + u_n)}.$$

Since k is small, and $r_n (1 + u_n) = \frac{r_n}{q_n + r_n}$ and is therefore less than unity,

$$\therefore \frac{du}{dn} = \Delta u_n = kr_n u_n (1 + u_n) \text{ approximately.}$$

$$\therefore kn = \int_1^{u_n} \frac{du}{ru(1+u)} \quad \dots(1),$$

putting $u_0 = 1$, as in Part I. This can be evaluated as r is a known function of u . Under random mating when recessives are few $r_n = \frac{1}{(1 + kn)^2}$ approximately, so selection is very slow. It will be shown that with some systems of mating successive small values of r_n approximate to a geometrical series, so that selection is vastly more rapid.

PARTIAL SELF-FERTILISATION.

Let a proportion l of the population be self-fertilised, $(1-l)$ mated at random, where l may have any value from 0 to 1 inclusive.

$$\begin{aligned} \therefore p_{m+1} &= l(p_m + \frac{1}{2}q_m) + (1-l)(p_m + q_m)^2, \\ q_{m+1} &= \frac{1}{2}lq_m + (1-l)(p_m + q_m)(q_m + r_m), \\ r_{m+1} &= l(\frac{1}{2}q_m + r_m) + (1-l)(q_m + r_m)^2. \end{aligned}$$

Clearly $u_{m+1} = u_m$, and

$$q_m = \frac{2(1-l)u}{(2-l)(1+u)^2} + \left(\frac{l}{2}\right)^m \left[q_0 - \frac{2(1-l)u}{(2-l)(1+u)^2} \right].$$

So there is a rapid approach to equilibrium, when

$$\left. \begin{aligned} p &= \frac{u(l+2u-lu)}{(2-l)(1+u)^2} \\ q &= \frac{2(1-l)u}{(2-l)(1+u)^2} \\ r &= \frac{2-l+lu}{(2-l)(1+u)^2} \end{aligned} \right\} \dots(2.1).$$

During selection

$$\left. \begin{aligned} kn &= \int_1^{u_n} \frac{(2-l)(1+u)du}{u(2-l+lu)} \\ &= \log_e u_n + \frac{2}{l} \log_e \left(\frac{2-l+lu_n}{2} \right) \\ r_n &= \frac{2-l+lu_n}{(2-l)(1+u_n)^2} \end{aligned} \right\} \dots(2.2),$$

unless $l = 0$, when $kn = u_n + \log_e u_n - 1$.

When recessives are sufficiently few

$$(2-l)r_n = \frac{l}{u_n} = le^{-\frac{lk}{2+l}} \text{ approximately,}$$

so $\frac{r_n}{r_{n+1}} = 1 + \frac{lk}{2+l}$ approximately, and selection is rapid.

PARTIAL INBREEDING.

Let a proportion l of the population be mated to whole brothers or sisters, $(1-l)$ mated at random. Let matings occur in the following proportions:

Mating	Proportion	Producing offspring	Matings of inbred offspring
$AA \times AA$	a_m	$a_m AA$	$la_m (AA \times AA)$
$AA \times Aa$	$4\beta_m$	$2\beta_m (AA + Aa)$	$l\beta_m (AA \times AA + 2AA \times Aa + Aa \times Aa)$
$AA \times aa$	$2\gamma_m$	$2\gamma_m Aa$	$2l\gamma_m (Aa \times Aa)$
$Aa \times Aa$	$16\delta_m$	$4\delta_m (AA + 2Aa + aa)$	$l\delta_m (AA \times AA + 4AA \times Aa + 2AA \times aa + 4Aa \times Aa + 4Aa \times aa + aa \times aa)$
$Aa \times aa$	$4\epsilon_m$	$2\epsilon_m (Aa + aa)$	$l\epsilon_m (Aa \times Aa + 2Aa \times aa + aa \times aa)$
$aa \times aa$	ζ_m	$\zeta_m aa$	$l\zeta_m (aa \times aa)$

Where

$$\begin{aligned}
 \alpha_m + 4\beta_m + 2\gamma_m + 16\delta_m + 4\epsilon_m + \zeta_m &= 1, \\
 \therefore p_m &= \alpha_m + 2\beta_m + \gamma_m, \quad p_{m+1} = \alpha_m + 2\beta_m + 4\delta_m, \\
 q_m &= \beta_m + 8\delta_m + \epsilon_m, \quad q_{m+1} = \beta_m + \gamma_m + 4\delta_m + \epsilon_m, \\
 r_m &= \gamma_m + 2\epsilon_m + \zeta_m, \quad r_{m+1} = 4\delta_m + 2\epsilon_m + \zeta_m, \\
 \alpha_{m+1} &= (1-l)p_{m+1}^2 + l(\alpha_m + \beta_m + \delta_m), \\
 4\beta_{m+1} &= 4(1-l)p_{m+1}q_{m+1} + 2l(\beta_m + 2\delta_m), \\
 2\gamma_{m+1} &= 2(1-l)p_{m+1}r_{m+1} + 2l\delta_m, \\
 16\delta_{m+1} &= 4(1-l)q_{m+1}^2 + l(\beta_m + 2\gamma_m + 4\delta_m + \epsilon_m), \\
 4\epsilon_{m+1} &= 4(1-l)q_{m+1}r_{m+1} + 2l(2\delta_m + \epsilon_m), \\
 \zeta_{m+1} &= (1-l)r_{m+1}^2 + l(\delta_m + \epsilon_m + \zeta_m), \\
 \therefore u_{m+1} &= u_m.
 \end{aligned}$$

When equilibrium is reached we can suppress suffixes in the above, and find $\gamma = 4\delta$.

$$\therefore (1-l)pr = (4-l)\delta = (1-l)q^2 + \frac{lq}{4}.$$

But

$$\begin{aligned}
 pr &= \left(\frac{u}{1+u} - q \right) \left(\frac{1}{1+u} - q \right), \\
 \therefore lq &= 4(1-l) \left(\frac{u}{(1+u)^2} - q \right). \\
 \therefore p &= \frac{l + (4-3l)u}{(4-3l)(1+u)^2} \\
 q &= \frac{4(1-l)u}{(4-3l)(1+u)^2} \\
 r &= \frac{4-3l+lu}{(4-3l)(1+u)^2} \\
 \therefore kn &= \int_1^{u_n} \frac{(4-3l)(1+u)du}{u(4-3l+lu)} \\
 &= \log_e u_n + \frac{4(1-l)}{l} \log_e \left(\frac{4-3l+lu_n}{4-2l} \right) \\
 r_n &= \frac{4-3l+lu_n}{(4-3l)(1+u_n)^2} \\
 \end{aligned} \quad \dots(3.1), \quad \dots(3.2),$$

unless $l = 0$, when $kn = u_n + \log_e u_n - 1$.

When recessives are very few,

$$(4-3l)r_n = \frac{l}{u_n} = le^{-\frac{lkn}{3l-4}} \text{ approximately,}$$

so $\frac{r_n}{r_{n+1}} = 1 + \frac{lk}{4-3l}$ approximately, and selection is rapid.

PARTIAL ASSORTATIVE MATING.

We consider a population containing a proportion r of recessives, the sexes being in equal numbers and mating so conducted that while each zygote is mated

once and only once in a given period, the probability of a recessive mating with a given recessive is greater than that of its mating with a given dominant, and similarly for dominants. Let θ be the proportion of dominant \times recessive and recessive \times dominant matings, then that of matings between two dominants is $1 - r - \theta$, between two recessives $r - \theta$,

$$\therefore (r - \theta)(1 - r - \theta) = (1 + \lambda)\theta^2,$$

where λ is positive. In general λ is a function of r , but since $\frac{\lambda}{1 + 2\lambda}$ is the coefficient of association as defined by Yule (3), between the phenotypic characters of spouses, and such coefficients are found to be valuable even when the proportions of the different classes vary greatly, it is probable that λ varies rather little with changes in the population. In a case of human assortative mating given by Yule $\lambda = 0.18$.

$$\begin{aligned} \theta &= \frac{\sqrt{1 + 4\lambda r(1 - r)} - 1}{2\lambda}, \\ \therefore p_{m+1} &= \frac{(p_m + q_m)^2}{1 - r_m} - \theta_m \left(\frac{p_m + q_m}{1 - r_m} \right)^2, \\ q_{m+1} &= \frac{q_m(p_m + q_m)}{1 - r_m} + \theta_m \left(\frac{p_m + q_m}{1 - r_m} \right)^2, \\ r_{m+1} &= r_m + \frac{q_m^2}{(1 - r_m)} - \theta_m \left(\frac{p_m + q_m}{1 - r_m} \right)^2. \end{aligned}$$

$\therefore u_{m+1} = u_m$; and, at equilibrium,

$$\left. \begin{aligned} p &= \frac{u}{1 + u} - q \\ \lambda(1 + u)^4 q^4 + u^2 (1 + u)^2 q - u^3 &= 0 \\ r &= \frac{1}{1 + u} - q \end{aligned} \right\} \quad \dots(4.1).$$

During selection

$$\begin{aligned} \frac{du_n}{dn} &= kr_n u_n (1 + u_n), \\ \therefore \lambda \left(1 - \frac{1}{ku_n} \frac{du_n}{dn} \right)^4 - \frac{u_n(1 + u_n)}{k} \frac{du_n}{dx} + u_n^2 &= 0. \\ \therefore kn &= \int_1^{u_n} \frac{du}{u - uf(\lambda, u)} \\ r_n &= \frac{1 - f(\lambda, u_n)}{1 + u_n} \end{aligned} \quad \left. \right\} \quad \dots(4.2),$$

where $f(\lambda, u)$ is the real positive root of

$$\lambda x^4 + u^2 (1 + u) x - u^3 = 0.$$

Clearly

$$\frac{u}{1 + u} > f(\lambda, u) > 0,$$

$$\therefore |u_n + \log_e u_n - 1| > |kn| > |\log_e u_n|$$

and

$$(1 + u_n)^{-1} > r_n > (1 + u_n)^{-2}.$$

Hence selection proceeds at a rate intermediate between those of equations (1.2)

and (2.3) of Part I. When recessives are few, so that u_n^3 is large compared with λ , $f(\lambda, u) = \frac{u}{1+u}$, approximately, and selection proceeds according to equation (2.3) of Part I. Hence the effect of partial assortative mating in speeding up selection is unimportant.

SELECTIVE FERTILISATION.

If λ has the same meaning as above, except that it applies to unions between gametes and not zygotes, as in Jones' (4) case, where λ was generally less than 100, though in one experiment it exceeded 10,000, equilibrium is reached in one generation, and $pr = (1 + \lambda) q^2$,

$$\left. \begin{aligned} \therefore p &= \frac{2\lambda + (1 + 2\lambda)u - \sqrt{(1+u)^2 + 4\lambda u}}{2\lambda(1+u)} \\ q &= \frac{\sqrt{(1+u)^2 + 4\lambda u} - 1 - u}{2\lambda(1+u)} \\ r &= \frac{1 + 2\lambda + u - \sqrt{(1+u)^2 + 4\lambda u}}{2\lambda(1+u)} \end{aligned} \right\} \quad \dots(5.1).$$

During selection,

$$\begin{aligned} kn &= \int_1^{u_n} \frac{2\lambda du}{u[1 + 2\lambda + u - \sqrt{(1+u)^2 + 4\lambda u}]} \\ &= \int_1^{u_n} \frac{1 + 2\lambda + u + \sqrt{(1+u)^2 + 4\lambda u}}{2(1+\lambda)u} du, \end{aligned}$$

and, if λ be constant,

$$\left. \begin{aligned} kn &= \log_e u_n + \frac{1}{2+2\lambda} [u_n - 1 + \sqrt{(1+u_n)^2 + 4\lambda u_n} - 2\sqrt{1+\lambda} \\ &\quad + (1+2\lambda) \log_e (1+2\lambda+u_n) \\ &\quad + \sqrt{(1+u_n)^2 + 4\lambda u_n} - \log_e (1+u_n) \\ &\quad + 2\lambda u_n + \sqrt{(1+u_n)^2 + 4\lambda u_n} \\ &\quad - 2\lambda \log_e 2(1+\lambda+\sqrt{1+\lambda})] \end{aligned} \right\} \quad \dots(5.2),$$

$$r_n = \frac{1 + 2\lambda + u_n - \sqrt{(1+u_n)^2 + 4\lambda u_n}}{2\lambda(1+u_n)}$$

Here again selection occurs at a rate intermediate between that of equations (1.2) and (2.3) of Part I, and when recessives are few $r_n = \frac{1+\lambda}{u_n^2} = \frac{1}{(1+\lambda)k^2n^2}$ approximately, so selection is only very slightly more rapid than during random mating.

DISCUSSION.

Effects similar to those produced by partial brother-sister mating may be expected from less drastic types of inbreeding, e.g. mating of cousins. Such moderate degrees of inbreeding must occur in any population where neither zygotes nor gametes of both genders are very mobile. When recessives are sufficiently rare

any cause which promotes inbreeding, even of distant relatives, will enormously increase their number for a given gametic ratio, and will make u_n tend to vary as r_n^{-1} rather than $r_n^{-\frac{1}{2}}$, making u an exponential function of kn instead of being proportional to it. Assortative mating will have little effect. Thus, if recessives number one in a million, and if only one mating in a hundred is between whole brothers and sisters, more than one recessive in 400 will mate with another recessive. To attain a like result by assortative mating a recessive must be more than 2500 times as likely to mate with a recessive as a dominant. This would imply such obstacles to mating with a dominant that the first recessive to appear could never mate at all. Probabilities of this order may, however, occur in selective fertilisation. Hence inbreeding or self-fertilisation appears to be necessary in the early stages of selection of a recessive character if this process is to be fast enough to be an effective cause of evolution. They cannot be replaced by moderate degrees of selective mating or fertilisation.

SUMMARY.

Expressions (2.1), (3.1), (4.1), (5.1) are found for the composition of Mendelian populations subjected to partial self-fertilisation, inbreeding, assortative mating, or selective fertilisation, and equations (2.2), (3.2), (4.2), (5.2) derived for the effect of selection on such populations. The effect of selection is greatly increased by inbreeding and self-fertilisation.

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THE MECHANISM OF CELL-DIVISION

I. THE FORCES WHICH CONTROL THE FORM AND CLEAVAGE OF THE EGGS OF *ECHINUS ESCULENTUS*

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(With 19 Text-figures.)

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I. INTRODUCTION.

THE process by which a living cell divides into two parts has long been the basis of speculation. As far as I know, the theories put forward can be divided into two categories:

- (a) Those which attribute cell-division to a difference set up in the interfacial tension at different parts of the cell surface.
- (b) Those which postulate contractile or other mechanical properties to the astral radiations.

The first type of theory has been associated in recent years with the names of McClendon (11), Robertson (13) and Spek (15). These authors base their conclusions upon analogies drawn from the behaviour of oil drops floating or immersed in water. McClendon infers that cell cleavage is due to a reduction in the interfacial tension at the poles of the cell, Robertson on the other hand infers that the reduction takes place at the equator. For the details of these arguments reference must be made to the original papers, but it is here necessary to draw

attention to the fact that the models put forward will only work successfully under certain conditions. In the first place, in actual practice, an oil drop can only be made to divide by alterations of interfacial tension when (a) the drop is of a considerable size, (b) when the rate at which the differential surface tension develops is very rapid, and in order that this may be the case very powerful reagents must be used. If the drop of oil be very small, any difference set up in the interfacial tension at one point is rapidly transmitted over the whole surface, and only a momentary disturbance in the form of the drop is observed. In the case of a larger drop, cleavage only occurs when the alkali employed for the local change in interfacial tension is sufficiently strong to act with great rapidity; otherwise the whole surface comes into equilibrium before cleavage can occur. Now the living cell is extremely small in comparison to the oil drops used for such experiments, and the application of such reagents used by McClendon or by Robertson would immediately cause the death of the cell. Again, the process of normal cleavage is relatively slow; it may take at least a quarter of an hour. Further, there is no reason to suppose that the energy required to cleave an echinoderm egg is of a different order to that required to cleave a *Paramoecium*, and this (according to Mast and Root⁽¹²⁾) is of the order of at least 383 dynes per square centimetre. When we compare this with the differences in interfacial tension which can be set up at an oil/water surface, it is difficult to accept the view that there is any real comparison to be drawn between the cleavage of a single phase oil drop and that of a living cell.

Both Robertson and McClendon make one very important assumption. They assume that the surface of the living cell is of a liquid nature. Further, both authors leave their analogy at the point where the cleavage is just complete. Now, one of the most striking features of the fully cleaved cell is that the two resulting blastomeres show no tendency to fuse with each other. Newly cleaved oil drops fuse together readily as soon as they are again in contact. Such drops can only be prevented from fusing if a third phase be present which forms a protecting layer on the surface of the oil sufficiently strong to oppose the operation of surface forces. There is no evidence that either McClendon's or Robertson's experiments would succeed under such conditions.

Now it is well known that the form occupied by the blastomeres of developing eggs or of other cell systems bears a remarkable resemblance to that of certain non-living systems. Roux⁽¹⁴⁾ showed that, if certain conditions be fulfilled, isolated oil drops immersed in a non-miscible fluid such as water can be arranged in a form bearing a very striking similarity to the blastomeres of a frog's egg. The conditions, which are of great importance, are as follows:

- (1) The surface of the oil drops must be coated with some third substance which will not mix freely with either the oil or the surrounding water. If such a substance (e.g. a film of an insoluble calcium salt) is not present the drops rapidly fuse together.
- (2) The drops must be enclosed within a limiting membrane which is capable of compressing them. Otherwise, there is no tendency for the drops to adhere to each other, and they remain apart and spherical in form.

Neither Robertson nor McClendon has attempted to cleave an oil drop in such a way as to allow the resultant drops to fulfil the above conditions.

The second system, which bears a resemblance to the form of living cells, is, of course, provided by soap bubbles. The interfaces between contiguous cells obey, in general, Errera's Law. "A cellular membrane at the moment of its formation tends to assume the form which would be assumed under the same conditions by a liquid film destitute of weight." The advantage of this system over that employed by Roux is, that once two soap bubbles are in contact, they take on a stable form, and there is no tendency for the two components to separate from each other. In this respect they resemble the living system. Errera's Law has recently been applied by D'Arcy Thompson⁽⁴⁾ to a variety of living cell complexes; but, in some cases at any rate, I think there has been a tendency to assume that the only forces acting on the cell membrane are those due to its liquid nature, viz. the interfacial tensions between the surface of the cell and that of contiguous films. If two equal soap bubbles are brought into contact they assume the form whose section is shown in Fig. 1. The free surface of each film is part of a sphere. The angles between contiguous films are 120° , and the inter-cellular film has a diameter equal to $\cdot866$ of that of each bubble. In order to explain that this particular form is not that occupied by all living two-cell complexes in which the cells are equal in size, Thompson suggests that the tension between the cell contents and the cell surface is not always equal to that between the cell surface and its external environment. In other words the tension on each side of AB may be greater or less than OA or PA . Now, it is a simple matter of observation to show that even this modification only provides a rough approximation to the form of a two-celled stage in the development of an *Echinus* egg. The important point, at the moment, is, however, to notice that the soap bubble system is essentially a two-phase system, in which two discontinuous volumes of air are kept apart by a film of soap solution, and are also kept apart from the external atmosphere.

Before proceeding with a detailed comparison between the cleavage of living cells and that of any oil and water system it is necessary to consider whether there is any real comparison between such systems when the process of cleavage is complete. In other words, are living cells essentially two-phase systems in which the internal phase is separated from the exterior environment by a surface phase immiscible with both the one and the other? Secondly, if this surface phase is of a non-liquid nature is there any force (comparable to that exerted by the limiting container in Roux's experiment) which prevents the cells separating from each other? If the surface layer is of a liquid nature, why does the form of living cells differ from that of adjacent soap bubbles?

It is, however, first convenient to consider a slight modification of the soap bubble system. Under normal conditions in which the bubbles are of considerable

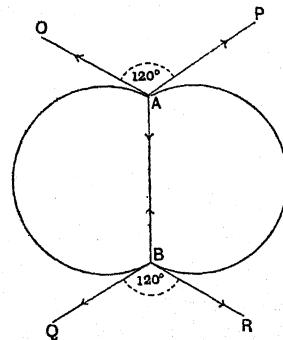


FIG. 1. Two equal soap bubbles in contact.

size, the thickness of the surface liquid phase is so thin that it is permissible to assume that the surface energy of the system is reduced to a minimum when the lines of contact between adjacent films form a well-marked angle of 120° , and the free surface of the bubbles are portions of spheres. Consider, however, the case in which the thickness of the surface film bears a much greater ratio to the size of the bubbles—in other words when the thickness of the liquid film is greatly increased. In this case the surface energy of the system will not be at its minimum when the free surface of the oil occupies the form of two partial spheres. Under these conditions, the free energy will be reduced by a flow of liquid from the poles of the system towards the equator, so as to reduce the area in contact with the external medium. This redistribution of the external phase upsets the resultant forces acting at the interface *AB* (see Fig. 1), and in order that equilibrium may be established the angle of 120° must be reduced. The new equilibrium is shown in

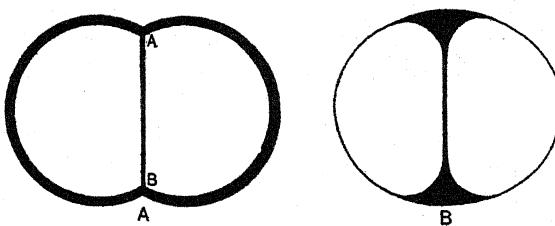


FIG. 2. Two water drops enclosed within a drop of olive oil. *A* is unstable, *B* is stable. Note the asymmetrical distribution of the oil phase, and the compressed form of the water drops in the stable condition. The oil phase is in black.

Fig. 2 *B* in which the internal phases are no longer two partial spheres but are obviously asymmetrical, due to pressure exerted along the long axis of the system.

This system can be fairly readily established by means of an oil and water system. If a drop of olive oil is immersed in a mixture of alcohol and water of the same specific gravity it is possible to inject into it by means of a fine capillary pipette two drops of the external watery phase. If some of the oil be now removed by means of the pipette, the effect on the form of the enclosed drops can be readily followed (Fig. 3).

The same changes in the form of the system can be effected with rather more difficulty by continuing to inject the watery phase into the enclosed drops, or by alternately injecting water and removing oil. It is obvious that it could be so performed that the volume of the whole drop remained unchanged.

The actual form of such systems depends upon the tension exerted by the outer surface of the oil, and upon the resistance of the two water drops to deformation by pressure. In this case these forces are probably both simply the expression of the interfacial tension between the oil and the water. It is important to notice, however, that an analogous system could be developed by means of solids if these were elastic. Consider the case of two spherical balloons enclosed within a third*.

* It is not necessary that the balloon containing the other two should be elastic, it must however be capable of exerting a pressure against the other two, and be capable of being deformed by them.

Now it is a fact that the compressed water drops in these experiments do bear a remarkable resemblance to the form of living cells, and I shall consider them again in more detail. They are essentially two-phase systems.

Let us now consider the living cell, in order to see whether or not it is legitimate to regard any of the above systems as a basis of comparison.

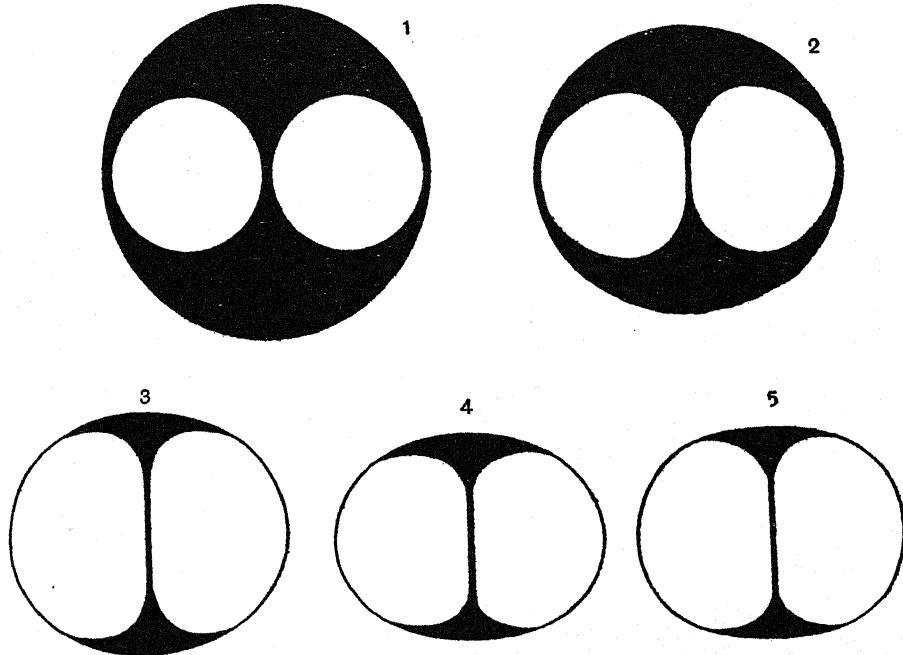


FIG. 3. Two water drops enclosed in a drop of olive oil. Note changes in the distribution of the oil and in the form of the water drops which occur when the relative volumes of water and oil are altered. The oil is black, the water white.

II. *The nature and development of the surface layer of the egg of Echinus esculentus.*

After the formation of the fertilisation membrane is complete the egg is almost perfectly spherical, and the cytoplasm appears to have a uniform structure throughout the whole egg. The most distinctive feature of the cytoplasm is the presence of a very large number of small granules, whose refractive index is considerably higher than that of the rest of the egg. The general impression gained is that these small granules or microsomes lie in a matrix in such a way as to give the appearance of an alveolar structure. The significant point is, however, that before and immediately after fertilisation the microsomes extend throughout the whole cytoplasm and can be seen close up against its peripheral boundary.

About a quarter of an hour after fertilisation, a marked change begins to occur at the egg surface. This surface which has hitherto remained perfectly smooth begins to develop a series of fine irregular processes. These processes are perfectly

transparent and free from microsomes; they gradually become more numerous and more obvious, but rapidly fuse with neighbouring processes so as to form a well-defined layer over the whole egg surface. Traces of the original radiate processes can frequently be seen, giving a striated appearance to the whole of this newly formed layer. This layer, which may be termed the "ectoplasm" of the cell, appears to be formed entirely from the matrix in which the microsomes are embedded, for although in the early stages of development of the ectoplasm a few microsomes are sometimes seen in its deeper regions, these microsomes soon disappear and the inner boundary of the ectoplasm is clearly marked off from the rest of the cytoplasm—or endoplasm. The term "endoplasm" is here applied to the region of the egg, crowded with microsomes, which lies beneath the ectoplasm.

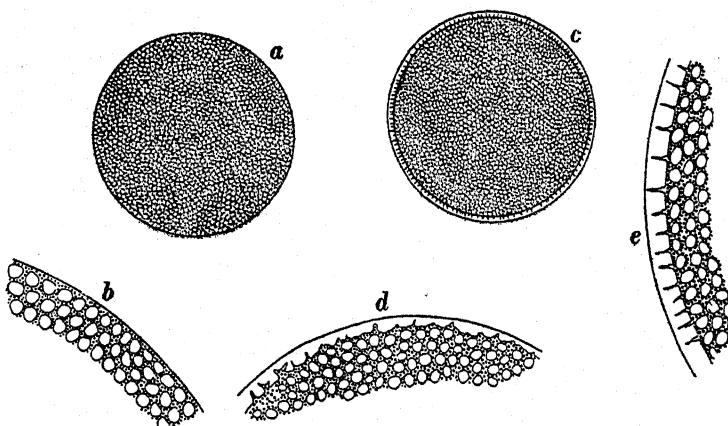


FIG. 4. (a) Unfertilised or newly fertilised egg of *Echinus esculentus* (fertilisation membrane and other membranes omitted). (b) Surface of unfertilised or newly fertilised egg enlarged. Note the small black micromeres lying near the surface. (c) Fertilised egg, 30 mins. after fertilisation. Note well-defined ectoplasm. (d) Surface of fertilised egg enlarged. Note the absence of micromeres beneath the surface, and irregular outline of endoplasm. (e) Surface of fertilised egg, when formation of ectoplasm is nearly complete.

The formation of this "ectoplasm" is not a new discovery. It was described by Andrews⁽¹⁾ in 1897 for the egg of *Arbacia*, and the existence of a well-defined superficial layer on developing eggs was noted by Hammar⁽⁷⁾ in 1896, and commented upon in 1908 by Goldsmidt and Popoff⁽⁵⁾. It has received the following names: hyaline layer, chorion, plamaschicht.

The formation of the ectoplasm is usually complete about half an hour after fertilisation, and its existence and behaviour can be readily followed up to the sixteen-celled stage. After its formation, no change takes place in the ectoplasm until the actual process of cell-division begins. At the moment when the equatorial region of the egg begins to straighten out in the formation of the segmentation furrow, it is seen (Fig. 5 B) that the ectoplasm in this region thickens, while at the same time it becomes distinctly thinner at the poles of the cell. As the segmentation furrow deepens, this redistribution of ectoplasm becomes more and more marked; as the furrow becomes complete the two resulting masses of endo-

plasm are clearly separated by a tongue of ectoplasm which eventually forms a complete inter-endoplasmic membrane. At the same time this membrane is continued over the whole surface of each blastomere. Whilst the bulk of the ectoplasm is thus distributing itself as if it were a fluid immiscible with the endoplasm, at the equator of the fully divided egg the outer surface of the ectoplasm shows very well-marked wrinkles, showing its solid nature*.

One point is clear therefore. At the surface of the cell there is a well-defined liquid layer which is immiscible with the underlying cytoplasm, and which in contact with the external sea-water tends to become solid.

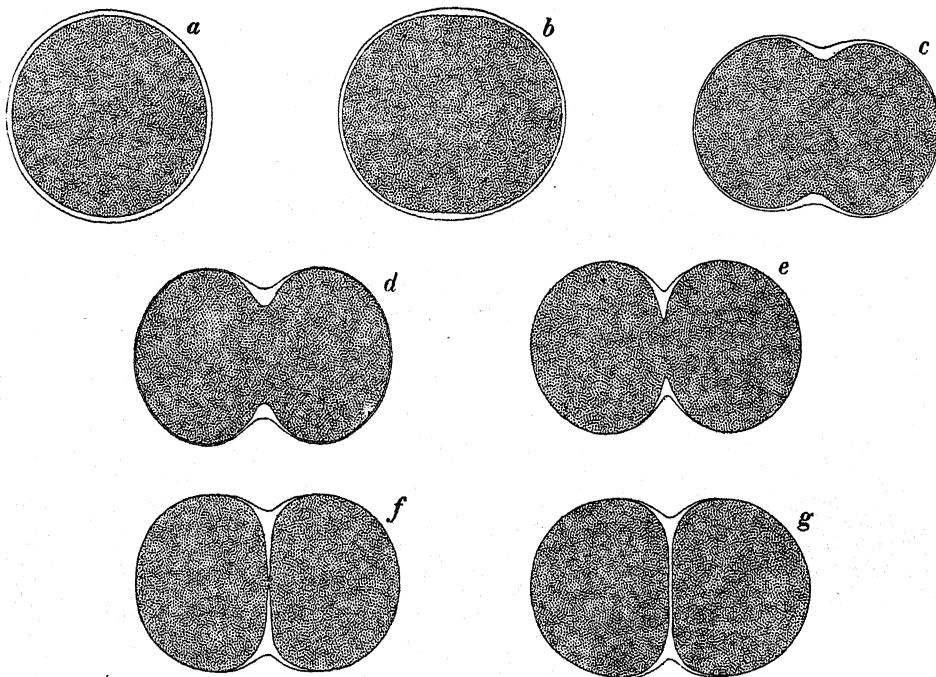


FIG. 5. (a-g) Normal cleavage of egg. Note the gradual flow of ectoplasm from the poles of cell to the equator. In (f) note that the endoplasm is almost completely divided. In (g) the ectoplasm has joined in the centre and the two masses of endoplasm are completely divided off from each other but are completely surrounded by ectoplasm.

III. The forces which determine the shape of the fully divided egg.

If we compare the form of the endoplasm in the fully divided cell and the distribution of the ectoplasm with the water drops and the oil in Fig. 1, the similarity is obvious. Now, in the latter system, we know that the form of the two enclosed water drops is directly due to the presence of the oil, and if the living cell is truly comparable to such a system, then the form of the fully divided cell should be due to the presence and properties of the "ectoplasm." Definite proof that this is so can be given as follows.

* The ectoplasm of the eggs of *Echinus miliaris* is much thinner and less obvious than that of *E. esculentus*. It is, however, of essentially the same nature.

If the fully divided cells are put into calcium-free sea-water, the cells separate from each other, as was shown by Herbst⁽⁹⁾. What is equally obvious, however, is the fact that the separated blastomeres are invariably spherical, and no longer retain their characteristic form (Fig. 6). Now, if the surface of the eggs is carefully examined during this process, it is found that the ectoplasm rapidly becomes unstable in the absence of calcium. Its well-defined outer surface disappears and the surface of the endoplasmic masses becomes covered with very fine radiate processes. The ectoplasm between the two masses of endoplasm is the last to dissolve, and gives rise to the "debris" seen in Herbst's diagrams. The dependence of ectoplasmic stability on the presence of calcium is also seen during the course of its development. If normally fertilised eggs, soon after fertilisation, are placed in sea-water containing no calcium, no definitive ectoplasm is formed. The whole surface of the egg becomes covered with very fine clear radiate processes, and the development of the egg soon stops. In the absence of calcium, the material which forms the ectoplasm fails apparently to acquire the property which prevents it mixing with the sea-water. This is borne out by a simple experiment. If a living egg in sea-water is cut across by a fine needle the cytoplasm remains as a sticky mass, often adhering to the point of the needle. If on the other hand the egg is cut whilst in a solution free from calcium, such as NaCl, the whole of the endoplasm flows out in a stream which is obviously miscible with the water. Similar properties can be detected by squashing the cell in the different solutions under coverslips. It, therefore, looks as though the external surface of the ectoplasm is essentially due to the effect of calcium on the material which normally flows out from the endoplasm when the ectoplasmic layer is formed.

The immediate point of importance is the fact that the departure from the spherical form on the part of the individual cells is due to the presence of a definite surface layer. That the presence of a limiting membrane round contiguous cells is capable of impressing upon them the form assumed by oil drops under similar conditions can be shown as follows. Under normal conditions the fertilisation membrane is sufficiently wide to prevent its interference with the cleavage of the egg (see Fig. 5). If, however, the unfertilised eggs are washed with acid sea-water (50 c.c. sea-water + 2 c.c. $N/10$ butyric acid) for a short period, and are then subsequently fertilised in normal sea-water, the normal fertilisation membrane is not formed. Under these conditions a fine membrane eventually forms close to the surface of the egg. The eggs cleave regularly but the form of the fully divided blastomeres is different from that of normal eggs. It will be seen that the form of these blastomeres is exactly that of the oil drops in Roux's experiments. A similar phenomenon occurs if for any reason the fertilisation membrane is sufficiently narrow to exert any pressure on the enclosed cells.

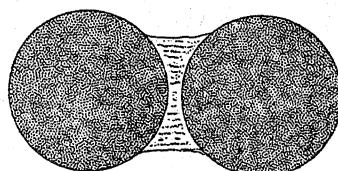


FIG. 6. Two-celled stage in calcium-free sea-water. Note remains of ectoplasm between the two spherical masses of endoplasm.

IV. Difference in properties of ectoplasm and endoplasm.

It has now been shown that there is, under normal conditions, a limiting membrane round the living cell which is immiscible with the external sea-water and which is the direct cause of the characteristic shape of the cells. Further, that this shape is due to pressure. Before finally accepting the view that Roux's experiments form a real basis of comparison to the living cell, it has still to be considered to what extent the surface ectoplasmic layer is immiscible with the internal or endoplasmic phase. The relationship between these two phases can best be analysed by a study of their respective physical properties.

Perhaps the most distinctive difference between these two phases lies in their different properties in respect to water. As already stated, unfertilised eggs possess no visible ectoplasm, and when they are exposed to hypertonic sea-water (50 c.c. sea-water + 10 c.c. $2\frac{1}{2}$ Mol. Van 't Hoff's solution) they shrink markedly and often irregularly: there is no evidence of any difference in the appearance of the periphery

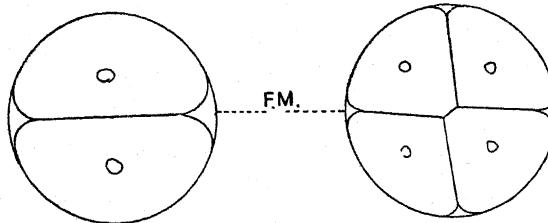


FIG. 7. Segmentation of egg when the fertilisation membrane remains close to the egg surface. Note the similarity in the form of the blastomeres to Roux's oil drops.

of the cell from the rest of the cytoplasm. The microsomes are uniformly distributed throughout the whole egg. If the eggs are put back into normal sea-water they rapidly regain their normal shape and size. From this we may infer that the whole cell is permeable to water but relatively impermeable to salts, and that its surface is of a more or less inelastic nature. Fertilised eggs, which have developed a visible ectoplasmic layer, when placed in a corresponding hypertonic solution show, however, a marked contrast. The following changes occur:

- (1) There is only a slight reduction in the volume of the whole egg.
- (2) The endoplasmic phase, however, shrinks markedly but regularly and remains spherical.
- (3) The ectoplasmic layer, on the other hand, becomes markedly wider than is normally the case. It also retains its regular outline.

There is thus a clear difference between the ectoplasm and the endoplasm. In hypertonic sea-water the former absorbs water and increases in bulk, the latter loses water and decreases in bulk. These properties are illustrated in Fig. 8.

If these fertilised eggs are retransferred to normal sea-water it is important to notice that the original relationships of the ectoplasm and endoplasm are not readily regained. This suggests that the changes induced by the hypertonic water are not entirely due to osmotic pressure. This is confirmed by the fact that the

uptake of water by the ectoplasm in hypertonic sea-water is completely abolished by the presence of acid. If the original hypertonic solution be acidified the whole egg decreases considerably in volume, and although the outer surface of the ectoplasm becomes more obvious as a definite membrane, the phase as a whole does not swell. Further, if the ectoplasm be swollen in hypertonic sea-water and then transferred to acid sea-water the ectoplasm at once loses water and becomes closely applied to the egg surface. Finally, if normal eggs are placed in acid sea-water the ectoplasm at once loses water and becomes extremely thin.

Although these phenomena require further investigation, they show fairly clearly that the ectoplasmic layer possesses properties not shared by the rest of the cell. It looks as though the amount of water held by the ectoplasm depends largely on the conditions which determine the uptake of water by imbibition. The amount of water held by the endoplasm depends, however, on the osmotic pressure of the external medium. It is interesting to note that these phenomena find an almost exact parallel in certain marine plant cells.

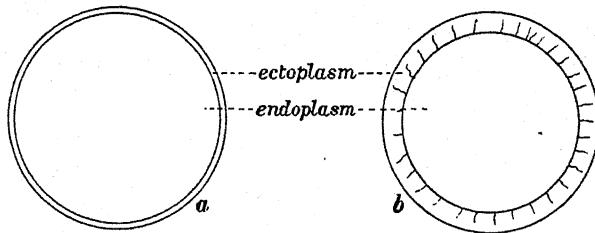


FIG. 8. (a) Fertilised egg in normal sea-water. (b) In 50 c.c. sea-water + 10 c.c. $2\frac{1}{2}$ M. NaCl. In (b) note contracted endoplasm, swollen ectoplasm, and fine processes extending into the latter from the endoplasm.

The nature of the forces operating at the surface of plant cells has recently been discussed by Kolte(10) and by Walter(16). The plant cell differs from the animal cell in that it is bounded on the outside by a tough resistant cuticle of cellulose. Separating the cuticle from the protoplast lies a membrane consisting of "pectin." In the case of newly formed cells, the form of the cell is determined by this pectic membrane, and it is this substance which binds individual cells together. Both Kolte and Walter have shown that when plasmolysis occurs in certain marine algae, the pectic membrane swells in a marked manner. Walter concludes that the total volume of the whole cell is limited by the volume enclosed by the external cuticle. The relative volume of the pectic membrane to the volume of the protoplast and cell-sap is determined by (a) the osmotic pressure exerted by the cell-sap (since the protoplast acts as a semipermeable membrane) which tends to increase the volume of the cell-sap, (b) the affinity of the pectic membrane for water, which tends to hold water and so limit the volume of the enclosed protoplast and cell-sap. Thus in a turgid cell the protoplast and the pectic membrane exert a pressure on each other. Any treatment which tends to reduce turgidity allows the pectic membrane to swell. An increase in turgidity, on the other hand, tends to a diminution in the volume of the pectic membrane, and an increase in the pressure exerted on the enclosed protoplast.

The only objection to this view is that both Kolte and Walter have shown that the increase in volume of the pectic membrane which accompanies plasmolysis is not readily reversible. Walter explains this by reference to the fact that colloids tend to absorb water at a greater rate than they lose this imbibed water when the conditions are reversed.

There thus appears to be some similarity between the pectic layer of plant cells and the ectoplasm of animal cells. So far, the constitution of the animal ectoplasm is unknown; its staining reactions resemble the pectin of plants, since in methylene blue both acquire a typical violet tint. The general properties of the ectoderm resemble those of a soap or a protein salt.

It is hoped that further work will throw more light on these facts, but at the moment it is necessary to consider briefly their bearing in relation to the form of newly divided cells.

If, as soon as the first cleavage is complete, eggs are transferred to hypertonic sea-water, the ectoplasm at once swells, and at the same time the two endoplasmic masses at once become spherical (Fig. 9 *b*). In some way the loss of water from the

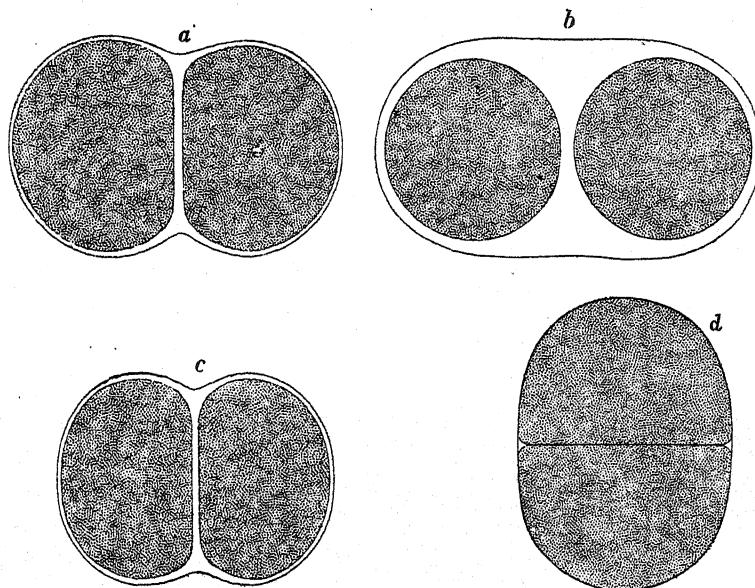


FIG. 9. (a) Cleavage just completed in normal sea-water. (b) Same egg transferred to hypertonic sea-water. Note swollen ectoplasm and contracted and spherical endoplasm. (c) Same egg in acid hypertonic sea-water. Note contraction of ectoplasm and change in form of endoplasm. (d) Final effect of acid hypertonic sea-water.

endoplasm relieves the pressure normally exerted by the ectoplasm so that the former is no longer compressed. The whole process can be compared to the oil and water system as follows. From a system such as Fig. 3, the volume of the enclosed water drops is reduced by removing some of the water, at the same time the volume of the oil phase is increased. The only difference between the two systems lies in the fact that the external surface at least of the ectoplasm of the living cell is of a solid nature and does not therefore allow its external form to approximate to that which would be the case were this surface a liquid.

When acid is added to the hypertonic solution the ectoplasm contracts violently owing to loss of water and again resumes its pressure on the enclosed masses of endoplasm. The first effect of the acid is therefore to cause the blastomeres to

resume their normal form, but very quickly further loss of water by the ectoplasm causes the pressure on the blastomeres to become abnormally high, and they begin to flatten against each other. The final result is shown in Fig. 9 *d*. The curious elongated form of the system is due to the solid nature of the external surface of the ectoplasm. As will be pointed out below, the ectoplasmic surface is extensible but not elastic. That the change of shape of the blastomeres in acid solutions is directly due to the effect of the solution on the ectoplasm is shown by the effect of acid upon cells in which the ectoplasm has been partially removed by treatment with calcium-free sea-water. This is shown in Fig. 10. It will be seen that acid only deforms the blastomeres where ectoplasm is present.

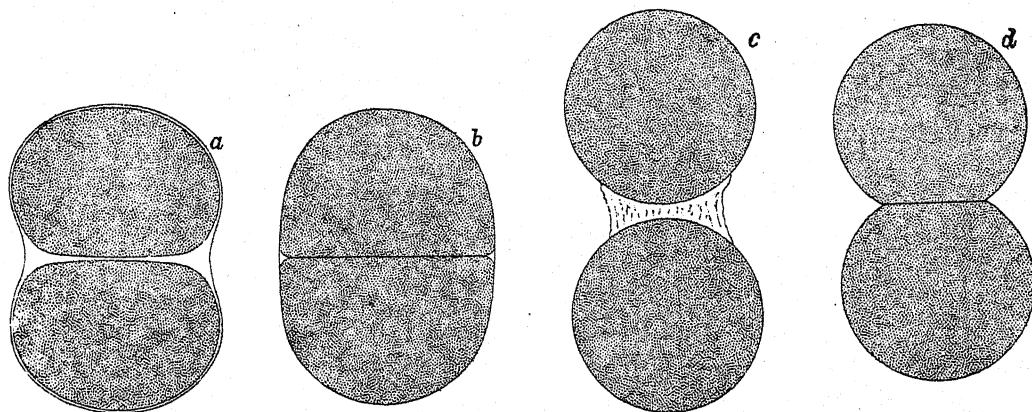


FIG. 10. (a) Normal egg in sea-water. (b) Same egg in acid sea-water. (c) Egg with ectoplasm partially removed by $\text{Ca}^{''}$ free sea-water. (d) Same egg in $\text{Ca}^{''}$ free sea-water + acid.

If fertilised eggs which have just developed a well-defined ectoplasm are treated with acid sea-water, this outer layer at once loses water and becomes much less apparent. After a short time the whole surface of the egg becomes covered with clear rounded vesicles. These vesicles are clearly derived from the ectoplasm. They do not form on unfertilised eggs or on eggs from which the ectoplasm has been removed by calcium-free sea-water. In eggs from which the ectoplasm has been partially removed they only develop on those regions still covered by ectoplasm. It appears as though, when the shrinkage of the ectoplasm reaches a certain point, the film becomes unstable, the outer solid layer ruptures and the fluid interior escapes to form droplets. The whole process is reversible as one might expect.

In a previous note⁽⁶⁾ dealing with the effect of acid on the form of the fully divided cell I was of opinion that the reagent acted by altering the surface tension between the external fluid and the surface of the cell. The fact that the external surface of the ectoplasm is of a solid nature makes such a suggestion untenable.

So far there seems firm ground upon which to regard the ectoplasm as a distinct phase from the underlying endoplasm; the living two-celled stage in the developing egg possesses, therefore, all the properties requisite for a comparison to an oil and water system, if we give to the external surface of the oil a solid extensible but not elastic membrane to make it equivalent to the external surface of the ectoplasm.

The relationship between endoplasm and ectoplasm is, however, of a more intimate nature than that between water and an immiscible oil. It has already been shown that during the development of the ectoplasmic layer fine strands of endoplasmic material extend out into the outer layer. These strands persist until just before cleavage, and can readily be seen by staining eggs with neutral red and treating them with hypertonic sea-water. The stain is readily taken up by the endoplasm, so that the processes extending into the ectoplasm are very distinct (see Fig. 11). During the process of cleavage (see below) these strands entirely disappear; they reappear again after each division is complete and can be seen joining individual blastomeres for many successive divisions. There is no actual continuity of ectoplasm and endoplasm in the sense that they are soluble or miscible with each other, but at the same time these processes may well indicate some physiological dependence on the part of the two layers of the cell. The existence of the intercellular connections between blastomeres was described by Andrews⁽¹⁾.

We are now in a position to state with some confidence that the form of contiguous blastomeres in a developing echinoderm egg is due to the existence of an external layer of the cell (which is here called the ectoplasm). This ectoplasmic layer is only stable in the presence of calcium, it has different properties from the endoplasm, and extends right round the whole egg and completely divides one mass of endoplasm from the other. The external surface of the ectoplasm has the properties of an extensible solid, the remainder is of a viscous fluid nature. Further, direct experimental data are now available to show that the analogy put forward by Roux is a real picture of the forces which control the form of contiguous cells. It is impossible to regard the form of living cells as directly comparable to that assumed by soap bubbles.

V. Behaviour and form of the asters during cleavage.

Having now established the distribution of the two phases of the cell during and after the process of division, it is possible to consider how this redistribution is effected. The simplest method of procedure is to consider under what conditions the spherical form of the undivided egg is lost, and what is the effect of each change in form on the distribution of ectoplasm and endoplasm.

The first tendency for the egg of *Echinus esculentus* to lose its spherical form can, under certain circumstances, be shown to occur about 30 minutes after fertilisation. At this time, no obvious change is observed if the eggs are kept gently agitated in water. If, however, the eggs are allowed to rest on the bottom of a flat vessel in such a way as to prevent one egg touching another, then the outline of the egg becomes irregular, and there is a marked tendency for the egg to flatten out on

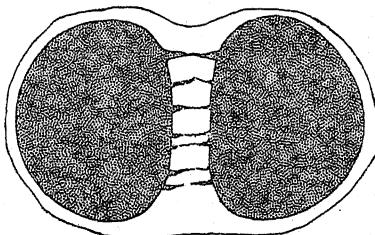


FIG. 11. Two-celled stage placed in hypertonic sea-water 15 mins. after completion of cleavage. Note inter-endoplasmic processes.

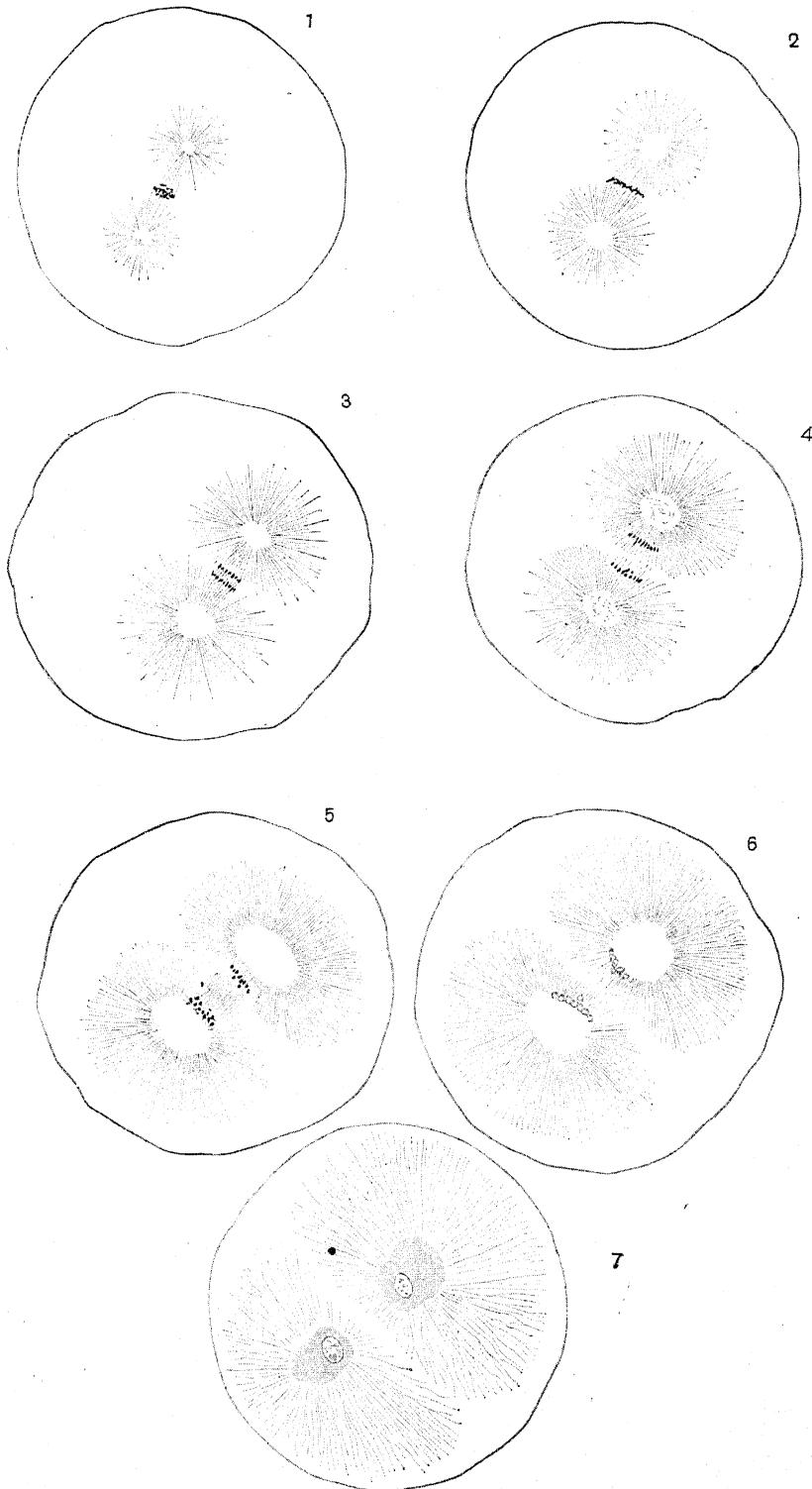


FIG. 12. Camera lucida drawings of developing asters from sections fixed in corrosive sublimate. Note change in shape of the asters after the anaphase stage is reached: also note loss of definition of astral rays with increase in the size of the asters.

the surface of the vessel. This irregularity in form is gradually lost if the eggs remain undisturbed. If the eggs are gently agitated, the spherical form is more rapidly regained. The addition of a little acid to the sea-water causes the eggs to become instantly spherical.

Sections of eggs which are undergoing this phase of irregular flattening show clearly that it is at this time that the whole of the endoplasm of the egg is pervaded by the enormous sperm aster. As soon as the sperm aster fades away, the irregularity in the form of the egg is lost. When the egg is resting at the bottom of the vessel, the astral rays appear to grow out from the sperm centrosphere along the lines of least resistance. They grow in a plane parallel to the glass, just as they grow in such a plane when eggs are made to segment under pressure. It will be shown later that this early departure from the spherical form is a special case of the rule that where astral rays approach the periphery of the cell the latter tends to be deformed in shape.

After the disappearance of the sperm aster there is no loss of form until the egg elongates just prior to the formation of the first cleavage furrow. In the following account of the cleavage, special attention will be directed to two points, (i) the form and distribution of the astral rays, (ii) the distribution of the ectoplasm. It must, however, be pointed out that whereas the form and distribution of the astral rays can satisfactorily be studied from sections of preserved cells, the distribution of the ectoplasm and the form of the cell must be studied in the living cell.

All the common fixatives contain acid, and this reagent at once disturbs the form of the cell. The only fixative not open to objection is neutral formalin, which therefore forms a convenient fluid in which to study the form of the endoplasm. In formalin the ectoplasm is partially destroyed, and so far sections of such eggs have not given satisfactory pictures of the astral rays.

The time between the fusion of the two pronuclei and the anaphase of the first division is marked by a gradual increase in the size of the two mitotic asters. As this increase in size takes place, it can be seen that each aster is an almost exact sphere, but as the spheres increase in volume the individual rays tend to become less distinct. This phase of increase in size of the two asters is illustrated by Fig. 12. At the anaphase stage (see Fig. 12, 3) the form of the asters begins to change. They continue to enlarge in volume but they are no longer spherical, they tend to flatten against each other in the median line (Fig. 12, 4-7). As soon as the two daughter nuclei are formed, practically the whole of the endoplasm of the egg is occupied by the two asters whose rays extend almost to the periphery of the endoplasm, and which are flattened against each other in the median line. So far the cell has retained its spherical outline, but at this moment there is a visible change in the shape of the whole egg. This can only be followed in the living cell. The cleavage furrow which now develops begins by a slight elongation of the polar axis so that the central region of the egg flattens, this process continues and the definitive cleavage furrow appears. Eventually, of course, the egg divides into two cells which show no tendency to fuse with each other. Now, the moment that the egg begins to elongate prior to the formation of the furrow a change takes place in the distribution

of the ectoplasm. Whereas this phase has, hitherto, been equally distributed over the whole cell, it now begins to flow from the poles towards the equator of the egg. This process continues so that when the cleavage furrow is well defined there is an obvious accumulation of ectoplasm in the furrow, while at the poles of the cell it is extremely thin. As soon as the egg is completely divided it can be seen that the two endoplasmic masses are completely divided by ectoplasm.

It has already been shown that the ectoplasm is responsible for the fact that newly formed blastomeres are not spherical in form, and there is no reason to suppose that its effect during the process of cleavage will differ in nature. Since the most obvious characteristic of dividing cells lies in the growth of the two asters, most authors are agreed that in some way these structures are associated with the act of cleavage. Evidence will now be presented to show that the form of the cleaving cell is the resultant of two forces, (i) the force exerted by the ectoplasm tending to maintain the spherical form, (ii) the force exerted by the growing asters which tends to elongate the axis of the cell, and eventually causes such a redistribution of ectoplasm that ultimate cleavage results.

VI. *The effect of the ectoplasm on the form of the cleavage furrow.*

We may first consider what is the effect of altering the normal pressure between the ectoplasm and endoplasm during cleavage. This can be done in two ways: it can be increased by withdrawing water from the ectoplasm by means of acids or by a mechanical membrane, or decreased by reducing the osmotic pressure of the endoplasm by hypertonic solutions. It has already been shown that the effect of acids applied during the phase of the segmentation furrow leads to a disappearance of the furrow, and if the furrow is not well developed, the whole egg regains its spherical form. The effect of a mechanical membrane tends on the other hand to prevent the initial elongation of the egg axis, although the cleavage furrow eventually forms. Whatever is the force tending to elongate the egg it is opposed by the action of the ectoplasm, since this always tends to maintain the spherical outline of the whole cell.

The disturbing effect of the ectoplasm on the form of the endoplasm can be eliminated (see above) by hypertonic solutions, so that if we apply such solutions during the actual process of cleavage we ought to be able to see what is the effect of the elongating force were it not opposed by the effect of the ectoplasm. The results of such treatment are shown in Fig. 13. It will be noted that the effect is invariably to increase the polar axis of the cell. Although the ectoplasm swells up in its characteristic way, it remains *in situ*. The endoplasm on the other hand shows an abnormal elongation, unless cleavage is complete, in which case each of the endoplasmic masses are perfectly spherical. Now, if the swollen condition of the ectoplasm be removed by acid the original and normal form of the cell is rapidly regained. That the abnormal elongation of the endoplasm is directly due to the removal of the pressure exerted by the ectoplasm is shown by the fact that if the ectoplasm be removed by means of calcium-free sea-water the form of the endo-

plasm is precisely similar to that in hypertonic solutions (see Fig. 14). It is therefore clear that the force which tends to elongate the egg axis is opposed (under normal conditions) by the presence of the ectoplasm.

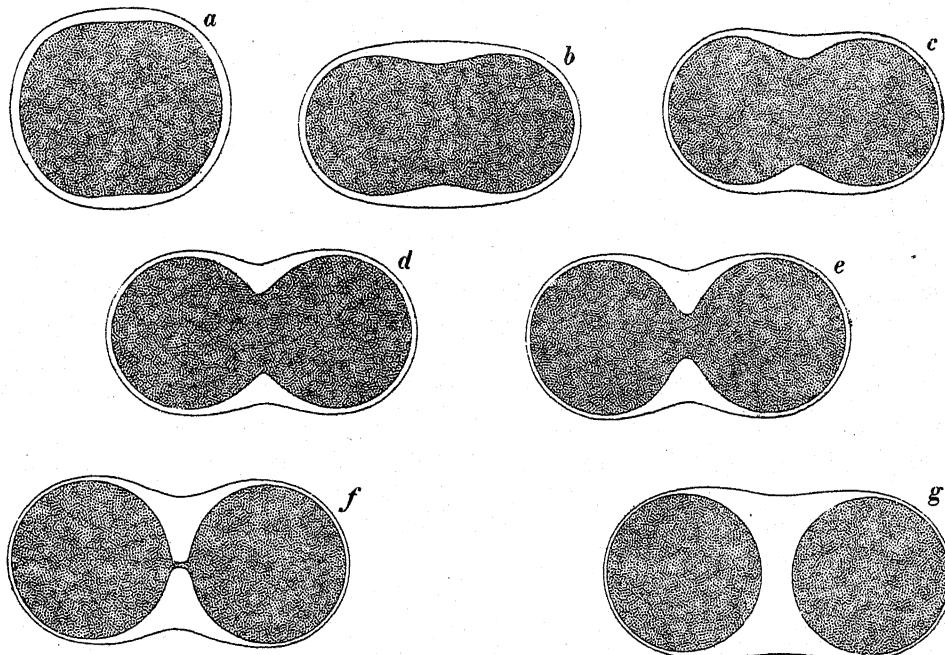


FIG. 13. Effect of hypertonic sea-water on the form of the cleaving eggs. Figs. 13 a-g show the effect of transferring the normal eggs shown in Figs. 5 a-g to hypertonic sea-water. Note change in form of endoplasm, with increased elongation of the polar axis. Note absence of inter-endoplasmic processes during cleavage.

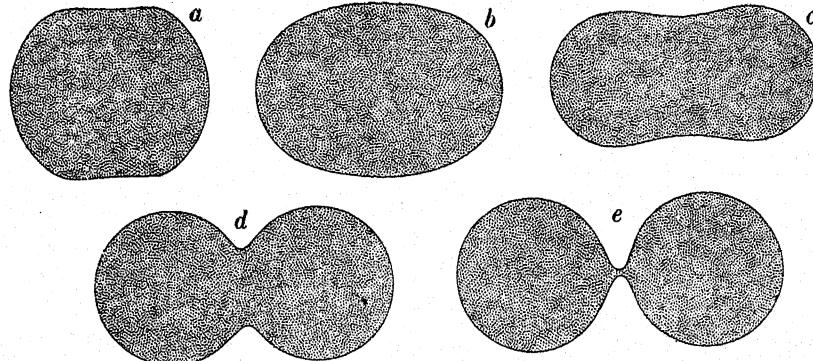


FIG. 14. Cleaving eggs in Ca^{++} free sea-water. Eggs transferred to the Ca^{++} free medium just before cleavage. Note absence or reduction of ectoplasm and similarity in form of endoplasm to that produced by hypertonic sea-water.

VII. *The relation between the size and position of the asters to the size and position of the cleavage furrow.*

Whenever astral rays approach within a certain distance of the periphery of the endoplasm they are capable of deforming the surface of the cell. This was shown in 1901 by Wilson (17). Again, cells such as echinoderm eggs do not cleave unless astral rays are in the neighbourhood of the periphery of the ectoplasm. This was also shown by Wilson, but since, in my opinion, it is a matter of considerable importance, it is possibly of value to offer confirmatory evidence. The most striking and direct proof is provided by the fact that the form and extent of the cleavage furrow depend upon the size and position of the asters. As is well known, a variety of reagents allows mitotic division of the nucleus to occur, but inhibits the asters from reaching their normal size. In such cases, the mitotic division of the nucleus occurs normally although the asters and the spindle remain abnormally small, and are often asymmetrically situated in the cell. Such nuclear division is not accompanied by cleavage of the cytoplasm unless the astral rays extend to the periphery of the cytoplasm. This phenomenon was described by Wilson. It is also figured below, in which case the normal development of the asters was originally interfered with by means of ether. Similar circumstances are often found in the normal eggs of other animals. For example, in Ctenophores—where the astral rays approach one side of the egg sooner than the other cleavage occurs from one side; also, to a more marked degree in the Crustacea—where cleavage is of a type comparable to Fig. 15 b.

Wilson showed that the normal astral radiations disappear if the eggs are exposed to a solution containing ether. On replacing the eggs in normal sea-water the radiations reappear: they do not, however, reach their normal size before again fading away. The result is that the egg may subsequently form a cleavage furrow, which fails to cleave the egg. As soon as the asters fade away all development of a cleavage furrow ceases. This experiment has been repeated, and Wilson's results have been confirmed.

If eggs of *E. esculentus* are allowed to develop in normal sea-water until the anaphase of the first division and are then transferred to 3 % solution of ether in sea-water, the astral rays very rapidly (2-3 minutes) disappear, and a clear irregular space appears in the centre of each of the original asters. If these eggs are now returned to normal sea-water, the asters reappear within about 15 minutes. In some of these eggs the asters rapidly extend to the periphery of the cell, and the latter cleaves normally into two cells. In other eggs, however, the reformed asters never extend to the periphery of the endoplasm, before fading away. In such eggs no cleavage occurs until the second nuclear division.

If eggs are allowed to develop in normal sea-water until the telophase stage is reached, and are then etherized, and returned to normal sea-water again, it is found that whereas two large asters existed at the beginning of the treatment, yet when the asters reappear in sea-water again, they appear not as two large asters but as

four asters much smaller in size. The two original daughter nuclei divide in conjunction with the four new asters and the cell divides into four normal blastomeres. In other words the first cleavage has been entirely omitted.

Finally, if eggs are etherized in 2.5% ether solution, and are then transferred to sea-water containing a very small concentration of ether, e.g. 0.05%, the asters which reform in the sea-water always remain small. Nuclear division occurs normally, but, owing to the small size of the spindle and of the asters, the nuclei remain close together and no cleavage occurs. The cell thus becomes a well-marked syncitium (Fig. 15 a). Eventually, however, the numerous small asters between them extend throughout the whole egg, and at this moment multiple cleavage occurs. In some eggs there appears to be a tendency for the nuclei with their asters to collect at the surface of the egg (see Fig. 15 b), and when the

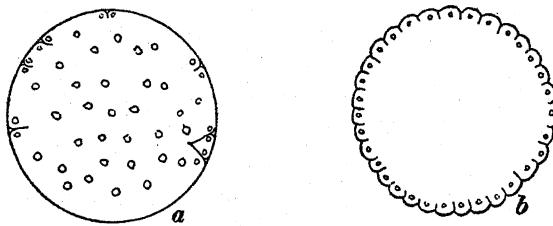


FIG. 15. Eggs segmenting in the presence of 0.05% ether: (a) note numerous nuclei forming syncitium, (b) note peripheral arrangement of nuclei and incomplete cleavage planes.

asters of these nuclei extend to the egg surface there is a distinct tendency for segmentation furrows to appear between them, although these furrows are never complete.

There can be very little doubt, therefore, that the nature and extent of the cleavage furrow are very closely associated with the position and the size of the asters. If, then, the appearance of a cleavage furrow is the direct mechanical effect of asters which extend to within a critical distance of the periphery of the endoplasm, and these asters are made to disappear before the cleavage furrow is complete, then further cleavage should cease and the egg should tend to resume its spherical form. This is the case. If eggs are allowed to develop in normal sea-water until the cleavage furrow is just beginning and are then etherized in 2.5% ether, the condition shown in Fig. 16 can be obtained. In these eggs a well-defined cleavage furrow exists but there are no asters. On transferring such eggs to sea-water, the cleavage plane is gradually lost.

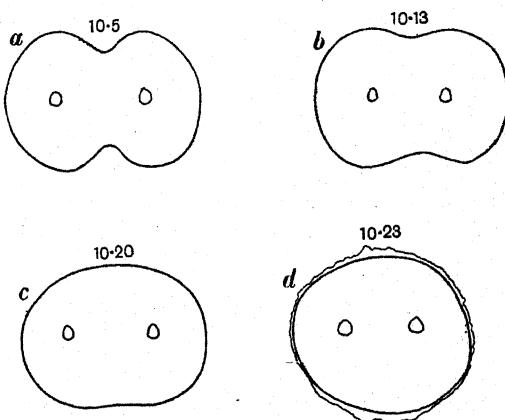


FIG. 16. Egg with normal cleavage furrow placed in 2.5% ether sol. for 20 minutes, then transferred to normal sea-water. Note absence of asters and gradual loss of cleavage furrow; also the crinkled ectoplasm in d.

If the original cleavage furrow was shallow, then on transference to normal sea-water after etherization, the egg gradually becomes completely spherical, at the same time the surface of the ectoplasm is thrown into distinct folds. If, however, the original furrow was well developed, then on return to sea-water the egg tends to retain an elongated form although the furrow itself disappears. At the same time the wrinkling of the ectoplasm is extremely obvious in the equatorial region. The elongated form of these eggs is however rapidly lost as soon as the asters for the next nuclear division begin to approach the periphery of the endoplasm.

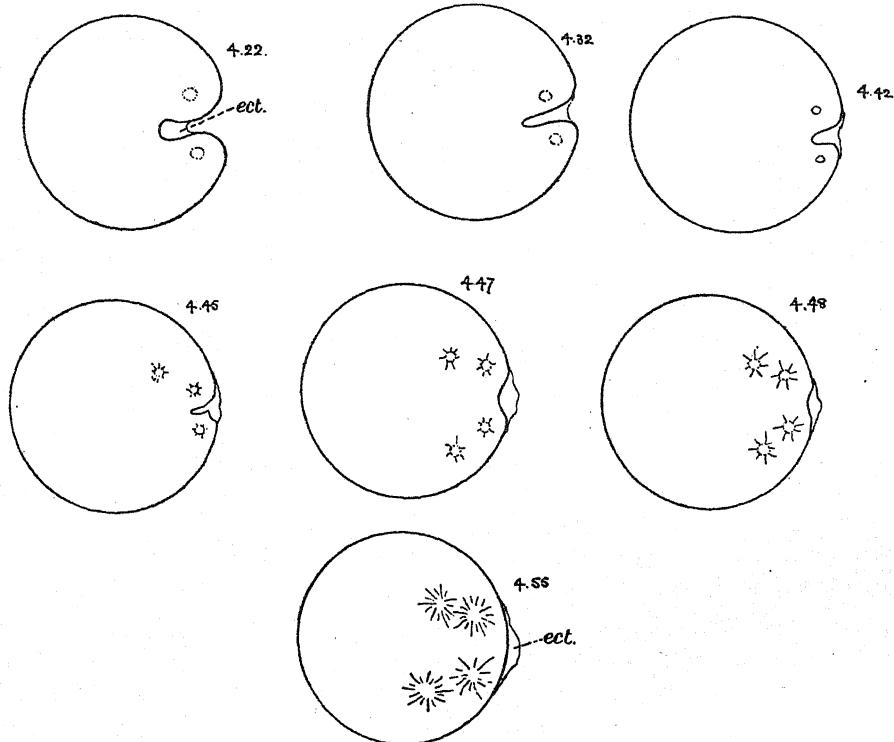


FIG. 17. Egg transferred from 2% ether to normal sea-water. Note asymmetrical astrospheres without astral rays, also cleavage furrow. Note gradual obliteration of cleavage furrow, and displacement of ectoplasm. Note subsequent division of astrospheres and development of astral rays.

Besides ether, many other reagents tend to inhibit the normal development of the asters, and yet allow the nucleus to divide normally. Among such reagents is slightly hyperalkaline sea-water. Figs. 18 *a* and *b* show an egg cleaving in 25 c.c. sea-water + 0.4 c.c. $N/10$ NH_4OH , the asters are small and asymmetrically situated. The result is a cleavage furrow on one side of the egg only. The same thing occurs in hypertonic sea-water. A deficiency of calcium, or potassium, has the same effect.

The form of the cleavage furrows in many of these reagents is extremely irregular (see Fig. 18). It is obvious that in such cells some irregularity exists between the ectoplasm and endoplasm which interdigitate with each other by fine strands of endoplasm. It is

almost impossible to conceive how such furrows could be the result of a differential interfacial tension at the poles and at the equator of the cell; they are, however, explicable on the assumption that the furrow is being brought about by a redistribution of the different phases of the egg.

Mechanical properties of the aster.

Before discussing the nature of the force which is exerted on the endoplasm by means of the asters, and which in conjunction with the force exerted by the ectoplasm brings about the normal form of cleavage furrow, it is of value to point out that the evidence from microdissection (Chambers⁽³⁾) and from the use of the centrifuge (Heilbrunn⁽⁸⁾) indicates fairly clearly that the region of the endoplasm occupied by the astral rays is of a more rigid or viscous nature than the non-radiate regions. The initial increase in the viscosity of the egg which takes place soon after fertilisation is directly associated with the existence of the fully formed sperm aster which pervades the whole egg. As soon as this aster fades away the cytoplasm again resumes the fluid state. Similarly, Chambers⁽³⁾ has shown that the asters during cleavage are areas of considerable rigidity as compared with the liquid surface regions of the cell.

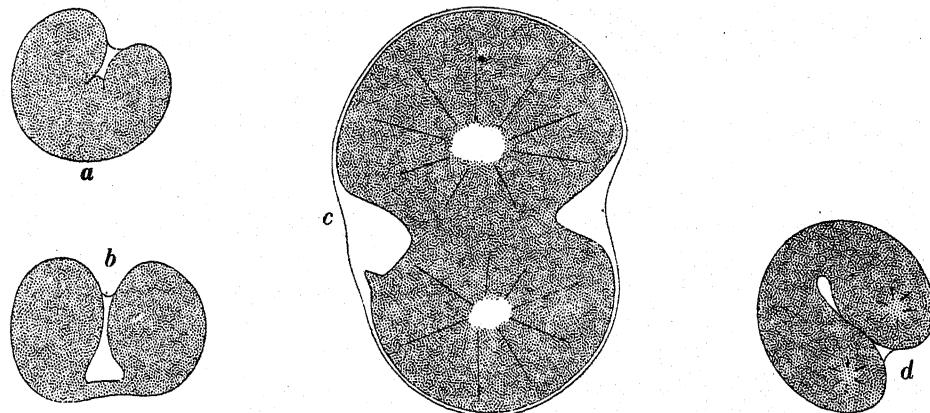


FIG. 18. (a) Cleavage of egg placed in 25 c.c. sea-water + 0.4 c.c. N/10 NH₄OH immediately after fertilisation. (b) Ditto. (c) Cleavage of egg placed in 50 c.c. sea-water + 2½ c.c. 2½ Mol. NaCl immediately after fertilisation. (d) cleavage of egg placed in potassium-free sea-water immediately after fertilisation.

VIII. *Discussion.*

The whole of the evidence concerning the forces which control the form of the cell during and after cleavage can be summarised as follows. Soon after fertilisation the protoplasm of the egg of *Echinus esculentus* is distributed into two distinct phases. The external phase or ectoplasm consists of a viscous fluid which, in contact with the external sea-water, develops a solid, extensible, but not very elastic film. The internal phase or endoplasm is characterised by the presence of numerous microsomes. It is the endoplasm which is responsible for the osmotic properties of the cell, and under normal conditions this osmotic force is opposed by the

surrounding ectoplasm. In the fully cleaved cell the two separate masses of endoplasm assume their characteristic form owing to the compression exerted on them by the ectoplasm. If the ectoplasmic pressure is released, the endoplasm is always spherical. Cleavage only occurs when there are asters present in the cell, and when these asters extend to the periphery of the ectoplasm. The nature and extent of the cleavage furrow depend on the size and on the position of the asters. The form occupied by the endoplasm during the process of cleavage depends upon the extent of the force exerted by the surrounding ectoplasm which tends to prevent the asters from elongating the egg. The asters are more viscous than the remainder of the endoplasm, but can be deformed by pressure.

The form of the fully divided cell can legitimately be represented by a liquid system of oil and water. The distribution of the ectoplasm and the form of the

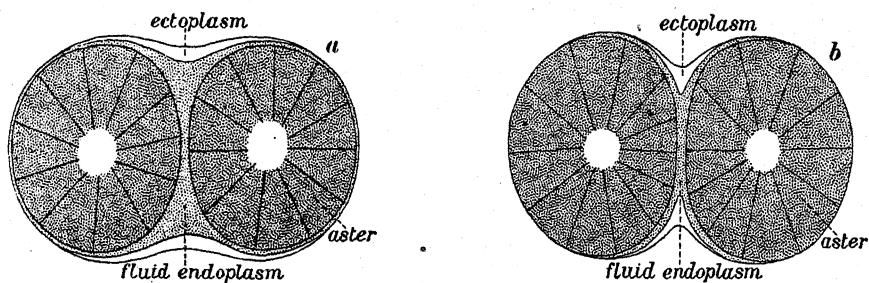


FIG. 19. (a and b) Diagram illustrating the redistribution of the cell phases caused by enlarging asters.

endoplasm of fully divided cells are exactly those of the oil and water, when a film of oil encloses two water drops and separates them from each other and from an external environment of water.

If a fairly large drop of olive oil be immersed in a mixture of alcohol and water of the same specific gravity, it is possible to inject into it two drops of the external medium. If these drops are gradually enlarged, the external surface of the oil remains spherical until the diameter of each of the enclosed and equal sized water drops is nearly half the diameter of the oil. If, now, the volume of the water drops be increased, or if the volume of the oil be decreased, a marked change in the form of the system takes place. The whole system elongates in its polar axis, the enclosed water drops flatten equatorially and are separated by a film of oil, and finally the oil flows away from the poles and collects at the equator as shown in Fig. 3*. In this model the enlarging water drops may be regarded as comparable to the asters, the oil representing the peripheral endoplasm plus ectoplasm. To make the analogy complete it is, of course, necessary to suppose that the enlargement of the watery phase takes place at the expense of the oil phase, but this assumption obviously presents no real objection to the nature of the forces involved. As long as the combined diameters of the two enclosed asters (or two enclosed water drops) are slightly less than the diameter of the endoplasm (or oil), no deformation of the

* The flow of liquid from the poles of the living cell to the equator is thus the direct effect of the force exerted by the asters, and is not in itself the cause of cleavage. Contrast this with Spek (15).

endoplasm (or oil) takes place. Also, up to this point the enclosed asters (or water drops) are spherical. As soon as this condition is passed then deformation tends to occur. In the case of the oil and water system, as soon as the combined diameter of the two water drops reaches this critical value the enclosed drops are pressed against each other in the median line; at the same time the oil flows away from the poles of the drop and collects at the equator. As the water drops increase in volume there comes a time when the pressure exerted by them is sufficient to rupture the thin film of oil left over the poles, and the whole system breaks down. If, in the case of the living cell, the ectoplasm is removed then the same changes in form occur as in the oil and water system. After the diameter of each aster approaches half that of the whole egg further increase in size of the asters (at the expense of the peripheral endoplasm) leads to a marked elongation in the polar axis, and a flow of peripheral endoplasm from the poles to the equator, as can be seen by the movement of the microsomes in some eggs. In the presence of the ectoplasm, however, the asters are not only compressed by the peripheral endoplasm, but also by ectoplasm, so that the elongation of the polar axis is reduced, and the asters are more conspicuously deformed from the spherical form*.

In the case of the egg, the normal cleavage furrow is only stable as long as the asters persist. If for any reason the asters disappear their substance becomes miscible with the peripheral endoplasm, and the egg tends to revert to the spherical condition. Under normal conditions, however, the asters do not disappear until practically the whole of the peripheral endoplasm is absorbed into the asters themselves, leaving the two masses held in a continuous film of ectoplasm which also completely separates them from each other. The asters then fade away, but the masses of endoplasm are not free to fuse with each other as they are separated by the film of ectoplasm with which they are immiscible, and which has, like the peripheral endoplasm, moved from the poles to the equator, and thereby reduced the free energy of the whole system.

In a preliminary paper (Gray(6)) it was suggested that the development of the segmentation furrow bore certain resemblances to a fluid drop held between two metal rings which gradually move away from each other, and data were put forward to indicate that the cleavage furrow of living cells begins to form when the distance between the centre of the asters was more than about .65 of the axis of the egg corresponding to the diameter of the supporting rings in the model experiment. Further consideration shows, however, that the model suggested is unsatisfactory. In the first place, experiment shows that a single phase oil drop can only be divided in a manner at all comparable to living cells if the oil on the outer side of the rings is unable to flow towards the centre of the figure. This can only be attained by replacing the supporting rings by flat plates whose edges are kept free from the grease which covers the rest of their surface. By such means a single phase oil drop can be cleaved, but the nature of the figure is unlike that of most living cells. The asters are obviously not comparable to flat plates, and the model is only justified by replacing the plates by spheres which can be distorted in form by pressure.

* In the case of the eggs of some *Echinoids* the force exerted by the ectoplasm is small, so that as long as the asters persist the newly formed blastomeres are nearly spherical. As soon as the asters fade the two blastomeres flatten against each other in the median line.

It has already been shown that if the present scheme be accepted, the elongation of the cell will tend to occur when the diameter of each aster is equal to about half the diameter of the egg*. At this stage the ratio of the distance between the centres of the asters and the axis measured in my previous publication is about .58, which is in reasonable agreement with the observed figures, since the latter figure makes no allowance for the delay caused by the resistance of the ectoplasm.

In considering the above system, it must be borne in mind that the external surface of the ectoplasm is solid. It is extensible and is not elastic, so that when once this ectoplasm has been elongated by the growth of the asters, it tends to prevent the endoplasm regaining its original form when the asters are removed from the partially cleaved cell. Also whereas the force exerted by the oil on the enclosed water drops is due to the interfacial tension between the oil and the external water, in the case of the cell it is due to the force required to extend the external solid surface of the ectoplasm and to overcome the viscosity of its inner liquid portion.

It is clear that since the asters are in fact areas capable of resisting pressure, and are more viscous than the peripheral endoplasm, cleavage of the cell must tend to be visible as soon as the diameter of each aster is half that of the whole cell and the pressure exerted by them overcomes that of the ectoplasm. The extent to which the egg will be deformed by elongation will depend upon the degree to which the asters resist deformation by pressure, and the degree to which the ectoplasm resists stretching†.

It may perhaps be pointed out that this analysis of the process of cell-division does not attribute any function to any part of the cell which is not susceptible of experimental treatment; at the same time it is not applicable in its simplest form to cells in which no visible asters are present.

Applying these conclusions to various types of cleavage, one might expect to find a definite range and type of variation to exist according to the relative degree to which the asters are symmetrically placed in the cell, and the degree to which the ectoplasm resists the tendency of the asters to remain spherical after they have reached the limiting size. The following variations can be accounted for and do actually occur:

- (1) Where the asters are unequal in size, cleavage is always unequal.
- (2) Where the asters are asymmetrically situated in the cell, cleavage is always asymmetrical, *e.g.* Ctenophores, Arthropods.
- (3) Where the ectoplasm is easily overcome by the asters, the initial elongation

* It will actually be slightly less than half, since the thin film of peripheral endoplasm at the poles and at the equator should not be included.

† The exact nature of each of the membranes seen on the surface of the eggs in different animals is difficult to define (see Carter (2)). It is obvious that each of these membranes will affect the form of the cleavage to a greater or less extent. If they are of a solid nature their effect will be proportional to their extensibility and elasticity. The arguments put forward in this paper are restricted to one of these membranes only, viz. the one which clearly develops on the external surface of the ectoplasm. In some eggs the ectoplasm is very thin, but if the arguments here put forward are correct, it must exist, since after cleavage the separated blastomeres do not fuse when in contact.

of the egg prior to cleavage will be well marked, *e.g.* Annelids, Molluscs, Actino-sphaerium.

(4) Where the ectoplasm is more resistant to extension, there is little or no elongation of the polar axis, *e.g.* Frog, Ctenophores.

(5) The "normal" type of equal cleavage as seen in *Echinus* eggs can be converted into any of the above types by appropriate interference with either the ectoplasm or the asters.

IX. Summary.

1. Soon after fertilisation the protoplasm at the surface of the egg of *Echinus esculentus* is differentiated off from the internal phase of the egg. The egg is thus divisible into two phases, the ectoplasm and the endoplasm.

2. Except during the process of cleavage fine processes of endoplasm extend out into the ectoplasm.

3. The outer surface of the ectoplasm is of a solid extensible nature. It is not markedly elastic. The underlying portion of the ectoplasm is fluid.

4. When cleavage occurs the ectoplasm tends to collect in the segmentation furrow and eventually divides the two portions of endoplasm from each other.

5. The normal form of the fully divided cell is due to the pressure exerted on the endoplasm by the ectoplasm.

6. The ectoplasm is only stable in the presence of calcium and potassium. It has different osmotic properties to those of the endoplasm. It rapidly shrinks in dilute acids.

7. During cleavage the normal form of the cleavage furrow is due to the force exerted by the ectoplasm against the force exerted by the asters.

8. The form and extent of the cleavage furrow depend on the position and size of the asters. The asters are capable of deformation by the ectoplasmic pressure.

9. Evidence is presented to show that the growth of the two asters must lead to a redistribution of the different phases of the protoplasm (peripheral endoplasm, and ectoplasm), such as is actually observed. Further, the final result of the enlargement of the asters must lead mechanically to normal cleavage. There are, thus, two essentials for cleavage, (i) the cell must be differentiated into two immiscible layers, ectoplasm and endoplasm, (ii) the cell must contain sufficiently large asters to bring about a redistribution of the endoplasm round these two centres.

10. The variations in the types of cleavage characteristic of other animal cells can be brought about in Echinoderm eggs by varying either the extent of the force exerted by the ectoplasm, or by varying the size or position of the two asters.

11. Cell-division in Echinoderm eggs is the direct result of the asters reaching a critical size.

In conclusion, I wish to express my gratitude to Mr R. Elmhurst, Director of the Marine Station, Millport, N.B., for providing me with facilities at Millport, and for his unfailing assistance in maintaining a constant supply of material during the period 1921-24.

The expenses of this research were defrayed in part by a grant from the Government Grant Committee of the Royal Society.

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Since this paper was prepared for the press (Aug. 1924) I have seen the account of cell-division given by Chambers in *General Cytology** (pp. 283-302). It is interesting to note that Chambers' account of the distribution of the different phases in the dividing egg is in essential agreement with that given in the present paper. He draws attention to the effect of the external "pellicle" of the egg and points out that this is capable of deforming the enclosed blastomeres. He also is of opinion that the elongation of the long axis of the cell is due to the growth of the asters, and that "when cleavage is imminent the entire periphery of the egg beneath its extraneous membranes is a flowing fluid. Within the egg the two growing asters of relatively high viscosity are separated from each other by a fluid equatorial zone."

The above article contains a reference to a paper by Just (*Amer. Journ. Phys.* **61**, 505, 1922) in which a description is given of the surface layer of the eggs of *Echinorachnius* during cleavage. The "hyaline plasma layer" of Just is obviously what is referred to in this paper as "ectoplasm." The behaviour of the two appears to be almost identical, but Just's interpretation of the facts differs materially from that given in this paper.

* University of Chicago Press, 1924.

THE EFFECT OF THE HYDROGEN ION CONCENTRATION ON THE BEHAVIOUR, GROWTH AND OCCURRENCE OF SPIROSTOMUM

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(Received 2 August 1924.)

(With Five Text-figures.)

THE SOURCE OF SPIROSTOMUM.

THE Spirostomum used in these experiments were obtained from three sources. The first source was a small pond on Sheep's Green, Cambridge. At the edge of this pond, where the water was no more than six inches to a foot deep, the bottom was thickly covered with leaves which had fallen from the willow trees overhanging the pond. Amongst these leaves Spirostomum could be found in vast numbers. Gently disturbing the leaves in certain places would cause a white cloud of Spirostomum to appear and, by dipping a jar into the water, great quantities of Spirostomum could be obtained. The number of Spirostomum thus obtained was so great as to render the water in the jar quite opaque. The actual numbers varied from 10 to 100 per c.c. The Spirostomum were first discovered here at the beginning of October, 1923, and they provided an undiminishing source of supply for six months. The Spirostomum obtained from this source were all *Spirostomum ambiguum* Ehr., and they are called in the experiments "wild forms."

The second source of supply was from an aquarium in the laboratory in which Spirostomum appeared in thick masses at the end of November and persisted during the winter months. These Spirostomum proved to be *Spirostomum ambiguum* Ehr. var. *minor*.

The third source of supply was from cultures of *Spirostomum ambiguum* Ehr. which were being maintained in the laboratory for experimental purposes. These I have termed "culture forms" in the experiments. Miss Bishop not only provided me with large numbers of culture forms for my experiments but also identified for me forms from Sheep's Green and from the aquarium. I owe her many thanks for this kindness.

THE pH OF THE SOURCES OF SUPPLY.

The pH of the water overlying the leaves in the pond on Sheep's Green was 7.4 at the beginning of October when the Spirostomum were first discovered. It varied slightly during the winter from 7.3 to 7.5. In the spring when the growth of aquatic vegetation started the pH rose above 7.5 to 7.6 and finally to 8.0. The rise to pH 7.6

coincided with the disappearance of the Spirostomum. If the water from among the leaves where the Spirostomum were clustered was carefully collected, then it could be shown that this water was slightly more acid than the overlying water, the difference being 0.1 pH.

The pH of the water in the aquarium was slightly higher than that of the pond, viz. 7.5 to 7.6. The aquarium contained masses of Spirogyra floating in the water in a not very active state of growth, and on the bottom was a deposit of mud two inches thick. A bacterial scum covered the surface. The aquarium stood in a window facing S.W. On sunny days the Spirogyra was sufficiently active to remove some carbon dioxide from the water by photosynthesis, and in consequence the pH would rise slightly. On these occasions I noticed that the Spirostomum were aggregated in clusters either on the Spirogyra or on the mud on the bottom. After a dull day or in the early morning there occurred an accumulation of carbon dioxide in the water as a result of which the pH fell slightly to 7.5 or 7.45, and on these occasions I noticed that the Spirostomum were not aggregated in clusters but were swimming freely in the water. They had, in fact, become planktonic and were no longer resting on the weed or on the bottom. I concluded from this that the Spirostomum became planktonic when the pH was 7.5 or slightly less, and increasing alkalinity, above pH 7.6, caused them to aggregate in clusters.

THE pH OF SPIROSTOMUM CULTURES.

The method of raising Spirostomum in cultures has been described by Miss Bishop (1923). She observed that it was necessary to use long narrow test-tubes for the cultures and that all attempts to cultivate Spirostomum in shallow dishes were unsuccessful. I ascertained the pH of a number of tubes containing flourishing cultures of Spirostomum and found them to be all from 7.4 to 7.6.

EFFECT OF pH ON SPIROSTOMUM.

Miss Bishop's observation that it was impossible to cultivate Spirostomum in shallow dishes recalls an earlier observation of Pütter (1903). Pütter found that when he placed Spirostomum in shallow watch glasses they all died. He demonstrated that this was not due to the concentration of the salts in the water by evaporation by exposing the watch glass in a damp atmosphere, when he found the Spirostomum died in the same way. On the other hand Pütter found that in sealed tubes the Spirostomum lived for a very long time. The explanation of this, according to Pütter, is that Spirostomum is extremely sensitive to the concentration of dissolved oxygen. In narrow tubes and in sealed vessels the oxygen concentration is reduced by the metabolic activities of the Spirostomum, and there is little or no surface where fresh oxygen from the air can diffuse into the water. In open dishes, on the other hand, the concentration of the oxygen dissolved soon reaches the saturation value of its partial pressure in air, and this concentration is, according to Pütter, fatal to the Spirostomum.

It is important to note that when the Spirostomum are allowed to die in these

shallow vessels they undergo certain malformations. As the animals become moribund the cilia beat slower and slower, finally ceasing altogether, first at the aboral end and then all over. As the cilia at the aboral end cease to beat, this end starts to swell and the animals assume a characteristic pear-shaped appearance. Fig. 1, which is copied from Pütter's paper, shows the pear-shaped malformation which he observed in *Spirostomum* dying after exposure in shallow watch glasses. I have myself observed the same appearances and can confirm Pütter's results, but I do not agree with his explanation.

The inability of *Spirostomum* to live in shallow dishes is due to the hydrogen ion concentration and has no relation whatever to the oxygen tension. This may be shown by the following experiments:

(1) *Spirostomum* are exposed in watch glasses in a damp atmosphere. One set of watch glasses contains the culture water of the *Spirostomum* (originally derived from Cambridge tap water), another set contains Cambridge tap water, and the third set contains Manchester tap water. Before placing the *Spirostomum* in the Cambridge or the Manchester tap waters they were washed free of their culture water by centrifuging two or three times. Twenty-four hours later it is observed that the *Spirostomum* in their own culture water and in the Cambridge tap water are moribund and malformed in exactly the same way as described by Pütter. Those in the Manchester tap water are normal in appearance and swimming freely in the water. In 36 hours the *Spirostomum* in the Cambridge tap water and in the culture water are all dead and have disintegrated. Those in the Manchester tap water continued to live for three weeks, at the end of which time they were perfectly normal in appearance and behaviour. Since the watch glasses are exposed side by side there can be no doubt that the oxygen tension in all the watch glasses is the same. Yet despite this they die in the Cambridge and thrive in the Manchester tap water. Clearly some other factor than the oxygen tension must be responsible for the death of the *Spirostomum*. It should be noted that Pütter does not record the source of the water used in his experiments. Probably it was all obtained from one source since he did not discover that the use of water almost free from dissolved carbonates would give quite different results from those he obtained.

(2) Some raw river water from the River Cam was diluted 1/10 with glass distilled water, and this diluted river water was used to dilute some Sörensen buffer mixtures. These diluted buffer mixtures were found to maintain a fairly constant pH when exposed to room air in a damp chamber. They did not appear to be poisonous to the animals. The concentration of the salts in the buffer solutions is no more than twice that of the culture solution and does not exceed a concentration that occurs commonly in hard waters. When the *Spirostomum* are first placed in these diluted buffer mixtures they writhe and twist for the first hour or so. The highest pH which I was able to obtain with these diluted buffer mixtures was 8.0, but this

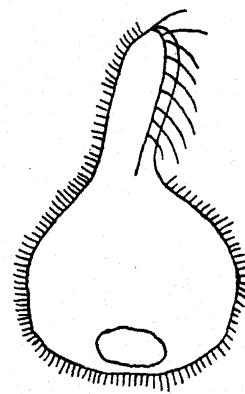


FIG. 1. Showing the pear-shaped appearance assumed by *Spirostomum*.

was sufficient to kill the Spirostomum after six hours' immersion. Moreover it is interesting to observe that the Spirostomum when dying in the diluted buffer mixtures of pH 8.0 do so in exactly the same manner as Pütter described and with the same pear-shaped malformation. Both wild specimens and individuals from cultures behaved in the same manner. In a diluted buffer mixture of pH 7.3 the Spirostomum were observed to be crawling slowly after six hours' immersion. Twenty-four hours later they were normal in appearance and were rising off the bottom and swimming in the water, which is one of the best tests for vitality in Spirostomum. In a diluted buffer mixture of pH 6.7 the behaviour of the wild and the culture specimens was somewhat different at first. After six hours' immersion the wild specimens were hardly moving, whereas the culture specimens were all moving actively. In a diluted buffer mixture of pH 6.2 both the wild and the culture forms almost ceased to move after six hours' immersion. After twenty-four hours all the specimens in both pH 6.7 and 6.2 started to crawl slowly round but never rose off the bottom of the watch glass. Although these specimens showed but little activity there was no sign of any swelling at the aboral end. I kept the Spirostomum in these diluted buffer mixtures in watch glasses exposed to a damp atmosphere so that no evaporation took place. At the end of this time only those Spirostomum in pH 7.3 were normal in appearance and behaviour. Those in pH 8.0 had died after twenty-four hours with the characteristic pear-shaped malformation ascribed by Pütter to too high a tension of dissolved oxygen. Those in pH 6.7 and 6.2 were crawling slowly with only the anterior cilia beating, but they were alive none the less and showed no sign of swelling in a pear-shaped fashion, even after a fortnight.

The concentration of the salts in all these watch glasses was in all cases the same, and since these watch glasses were exposed under a bell jar to the same moist atmosphere we may assume that the concentration of the dissolved gases in all is the same. The only difference between the water in the watch glasses is in the proportion of the phosphates, which provide the differences of pH . Assuming for the moment that it is the hydrogen ion concentration and not the variations in proportion of the phosphates that affects the Spirostomum, this experiment shows that in solutions of pH 8.0 Spirostomum are killed very rapidly and die in a similar manner to that described by Pütter when they are exposed in shallow watch glasses, that in solutions of pH 7.3 they flourish and attain their maximum activity, and that when the pH falls much below this value their activity is greatly retarded but they are not killed. When Spirostomum, whose activity has been retarded in this way, are removed into solutions of pH 7.3 they rapidly regain their normal appearance and activity.

In order to show that these effects are due to the hydrogen ion concentration and not to variations in the proportion of the phosphates, it will be sufficient to show that when the alterations in the hydrogen ion concentration are produced in a different way and not by buffering with phosphates, the effects on the Spirostomum are precisely the same. With this object some water from the River Cam was taken and fresh air aspirated through it which caused the pH to rise to 8.7. Some of this water of pH 8.7 was placed in a glass cell and some Spirostomum were added to it.

The final *pH* of the water in the cell was 8.4, the drop being caused by the water in which the Spirostomum were added containing considerably more carbon dioxide in solution than the aspirated water. The cell was sealed with a glass cover-slip. Here again a difference in the susceptibility of cultured and wild specimens was observed. The cultured forms survived for twenty-six hours but all became pear-shaped, the wild forms only survived six hours and assumed the pear-shaped form very rapidly indeed. After the experiment was finished the cell was opened and the *pH* of the water was tested. It was found to have fallen in extreme cases to 8.0, the drop in *pH* being caused by carbon dioxide evolved by the Spirostomum and other animals in the water. When the Spirostomum were sealed in raw River Cam water the *pH* of which had been adjusted to 7.4 by breathing into it and so increasing the tension of dissolved carbon dioxide they survived indefinitely. The production of a *pH* below 7.0 by means of carbon dioxide added to River Cam water is impossible without producing a lethal solution, so great are the quantities of carbon dioxide required to lower the *pH* below this value.

We have now established the following facts: (1) that solutions of *pH* 8.0 and above are toxic to Spirostomum and that in such solutions they die in a characteristic manner, (2) that solutions of *pH* 7.3 or thereabouts favour the activity of Spirostomum, (3) that solutions below *pH* 7.0 inhibit the activity of Spirostomum but are not lethal as are solutions of *pH* 8.0 and above. This provides us with a simple explanation of Pütter's experiments. Pütter must have used a hard water. Hard waters contain a sufficient quantity of the carbonates of calcium and magnesium in solution to give, when in equilibrium with the tension of carbon dioxide in room air, a *pH* of 8.0 or more. By exposing the Spirostomum in an open watch glass the tension of the carbon dioxide in the water would be reduced until it came into equilibrium with that of the carbon dioxide in the air of the room, this would cause the *pH* to rise sufficiently to kill the Spirostomum in the manner described. By using a soft water such as the Manchester tap water, which contains only a very small quantity of carbonates in solution (·0001 N for all carbonates, whereas a hard water varies from ·002 to ·01 N), we can expose this to the room air and the *pH* will rise no higher than 7.4. In this case, as I have already shown, the Spirostomum survive. Clearly therefore it is the *pH* and not the tension of dissolved oxygen which is responsible for the death of the Spirostomum.

The great importance of the hydrogen ion concentration in biological processes was not realised or even known when Pütter did his experiments, and it was unfortunate therefore that he did not try the crucial experiment of using a soft water. In order to prove his supposition that it was oxygen poisoning that killed the Spirostomum Pütter placed some Spirostomum in a hanging drop in a small chamber through which passed oxygen from a cylinder. He found that the Spirostomum died if treated in this way. My explanation of this is that the tension of carbon dioxide in the chamber was so lowered by the addition of the oxygen that carbon dioxide diffused out of the hanging drop and the *pH* in consequence rose to a lethal value. Pütter then substituted nitrogen for the oxygen in the chamber and he found that the Spirostomum survived in the hanging drop. But there is no evidence in his

paper that Pütter took any precautions to purify the gases he used. I myself have repeated the experiment, using nitrogen from a cylinder which has been passed through alkaline pyrogallol, and I found that it produced exactly the opposite result to that described by Pütter. When nitrogen purified in this way is admitted to a chamber in which the Spirostomum are confined so as to lower the oxygen tension, then I found that the Spirostomum died rapidly and with the same pear-shaped malformation as they did when the chamber was filled with oxygen. Nitrogen, when introduced into the chamber, causes a lowering of the tension of carbon dioxide in exactly the same way as the oxygen, and as this results in increasing the *pH* the results in both cases are identical. I would suggest that the nitrogen used by Pütter for his experiments was impure and contained as an impurity sufficient carbon dioxide to prevent the diffusion of this gas out of the hanging drop.

THE RELATION OF THE *pH* TO THE ACTIVITY OF SPIROSTOMUM.

The relation of the activity of the Spirostomum to the hydrogen ion concentration of the solution can be very simply demonstrated. Some hard water, such as Cambridge tap water, and some soft water, such as Manchester tap water, are allowed to stand in the air or have fresh air aspirated through them until the *pH* rises in the first case to 8.0 and in the second case to 7.2 or thereabouts. After treatment in this way 10 c.c. samples of these waters are placed in a number of test-tubes and to each test-tube is added different quantities of tap water saturated with carbon dioxide so that a series of tubes of graduated *pH* are obtained. The changes of *pH* in the tubes may be observed by adding indicators to each tube. The sulphon-phthalein indicators, in the very dilute concentrations required to show the differences of *pH*, are not poisonous to Spirostomum and do not appear to affect their vital activities in any way. The hard water provides us with a graduated series of *pH* from 8.0 down to 7.0 and the soft water with a series ranging from 7.2 down to 6.2. It is necessary to use the two different kinds of water since it is impossible, as has already been explained, to reduce the *pH* of a hard water much below 7.0 without adding a lethal concentration of carbon dioxide. Reference to a curve drawn from the figures given in a previous paper of mine (Saunders, 1923) will show that very small quantities of carbon dioxide are required to reduce the *pH* from 8.0 to 7.0, whereas the quantities required to reduce it below this latter value are relatively enormous.

To the graduated series of *pH* samples are added one or two drops of water in which Spirostomum have been thickly concentrated by centrifuging. The tubes are agitated in order to distribute the Spirostomum evenly and then the behaviour of the animals is observed. It will first be noticed that the Spirostomum sinks rapidly to the bottom of the test-tube, but that the rate of sinking is not the same in all the tubes. In those tubes where the *pH* exceeds 7.6 Spirostomum sinks very rapidly, in those tubes where the *pH* ranges from 7.0 to 7.4 the animals sink least rapidly, while there is a gradual increase in the rapidity of sinking as we pass from *pH* 7.0 to *pH* 6.0, but the rapidity of sinking at *pH* 6.0 is not nearly so great as at *pH* 8.0. It is evident that a slight increase in alkalinity above the optimum is far more harmful than a considerable increase in acidity. If the tubes be observed an

hour later it will be seen that the *Spirostomum* have risen off the bottom to a slight extent in pH 6.0 and that there is a gradual increase both in numbers and in the height to which they have risen as we pass up to pH 7.2 to 7.4, where the maximum is reached, then above this value there is a decline followed by a sudden cessation of activity as we pass to pH 8.0. For these experiments I made use of culture forms of *Spirostomum*, the wild ones seemed to dislike the change of water from their normal environment to that of the water in the test-tube. They do, however, behave in the same way as the culture forms, but since their activities are impaired by the change of water the results are not so striking. Culture forms give the same results if the changes of pH in the test-tubes are produced by the addition of small quantities of Sörensen's phosphates as buffers in the manner already described. But here again the results are not so striking because the activities of the *Spirostomum*, even the culture forms, are impaired by the change to the phosphate solutions, nevertheless

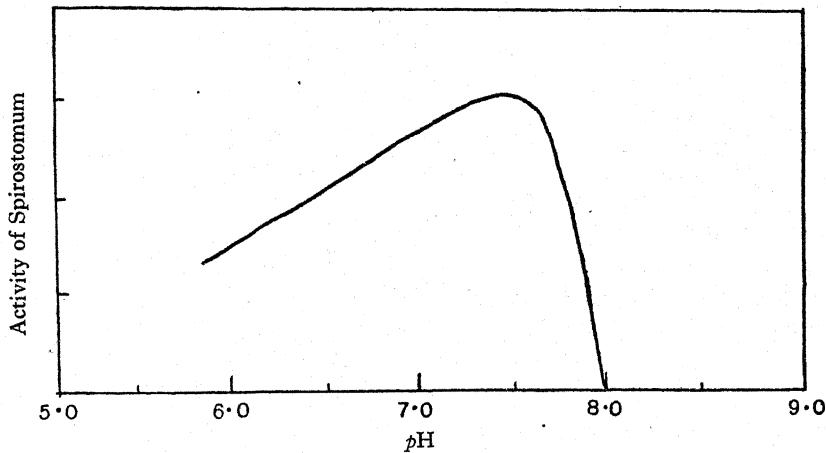


FIG. 2. Curve showing the relation of the activity of *Spirostomum* to the pH .

it is possible to obtain confirmation of the fact that it is the pH and not the carbon dioxide tension which is affecting their activity. Wild forms are so affected by the change into the dilute phosphate solutions, that they take 24-48 hours to recover, but after recovery they behave in the same manner as the culture forms.

The power of *Spirostomum* to rise from the bottom of the vessel in which they are living and to swim freely in the water is obviously derived from ciliary activity. We may therefore suppose that the general activities of the animal represent the changes in ciliary activity. This may be true for the pH values below 7.6, but above this value other factors which are produced by the increase in alkalinity, such as the greater permeability of the aboral end of the animal and the consequent characteristic pear-shaped malformation, cause the ciliary activity to decline much more rapidly than would be the case were it a direct effect.

The relationship of the activity of *Spirostomum* as measured by the height to which they rise in test-tubes to the pH of the water is shown graphically in Fig. 2. This curve has been compounded from the results of a number of observations made at different times.

The simplest method of demonstrating the effect of the hydrogen ion concentration on the activity of Spirostomum is to take 5 c.c. of some hard water in a test-tube and, after adding a few drops of cresol red, to shake the tube so as to bring the tension of the gases in solution in the water into equilibrium with the air. This will cause the *pH* to reach a value from 8.0 to 8.8, varying with the "hardness" of the water, and the cresol red indicator will assume a reddish purple colour. To another 5 c.c. of hard water in a test-tube add a few drops of phenol red and then breathe in the tube. On shaking the water in the tube will absorb carbon dioxide and the colour of the solution will become straw yellow. If now some Spirostomum be added to the water in both these tubes and the tubes shaken so as to distribute the animals evenly, afterwards standing them upright, it will be seen immediately that the Spirostomum in the more alkaline water (reddish purple) fall rapidly to the bottom, while those in the less alkaline or slightly acid water (yellow) continue swimming in the water or only fall very slowly.

EFFECT OF *pH* ON THE REARING OF SPIROSTOMUM IN CULTURES.

I tried to see whether the hydrogen ion concentration of the water had any effect on the rearing of Spirostomum in cultures. I used the methods described by Bishop (1923), but substituting for the pond or tap water used by her the diluted buffer mixtures. I found, however, that the phosphate mixtures in the dilution used were not sufficiently strong buffers to last for any length of time, so that the *pH* of the culture tubes did not remain constant long enough for the cultures to grow. The *pH* changed as soon as any growth of bacteria occurred, which was usually after four to six days. I am inclined to think that the phosphates were consumed or converted by the animal and vegetable life that developed in the test-tubes and that this resulted in the buffering effect being lost. Nevertheless, I have no doubt that the hydrogen ion concentration is an important factor in the culture of Spirostomum, for my experiments show that the activity of the animal is greatest within a narrow range of *pH* from 7.2 to 7.4, and both in flourishing cultures and in the pond where the animals occurred in enormous numbers the *pH* varied only between 7.2 and 7.6.

ATTRACTION OF SPIROSTOMUM INTO WATER OF CERTAIN *pH*.

Seeing that the plentiful occurrence of Spirostomum in nature and in cultures in the laboratory occurs only when the *pH* of the water varies within the limits 7.2 to 7.6 and, further, that experiments show that when the *pH* rises above 7.8 the animals are killed, while a reduction of the *pH* to 6.0 materially reduces their activity, it will be interesting to see whether the Spirostomum will move into water of any definite *pH* if they are given the opportunity. For this purpose I used narrow tubes .75 cm. in diameter and 20 cms. long which could be closed at each end by means of a cork. By means of a long capillary pipette the tube could be filled for half its length with a solution of one *pH* and then a solution of another *pH* run in on top. The two liquids mix slightly at the junction. If the tube be corked so as to exclude any bubbles of air, it can be tilted in any direction without any further mixing of

the liquids occurring. In order to observe the pH of the water in the tube it was coloured with a suitable sulphonphthalein indicator. If solutions of different pH in which *Spirostomum* are placed be pipetted into the tubes then we can easily observe the behaviour of the animals when confronted with water giving a choice of hydrogen ion concentration.

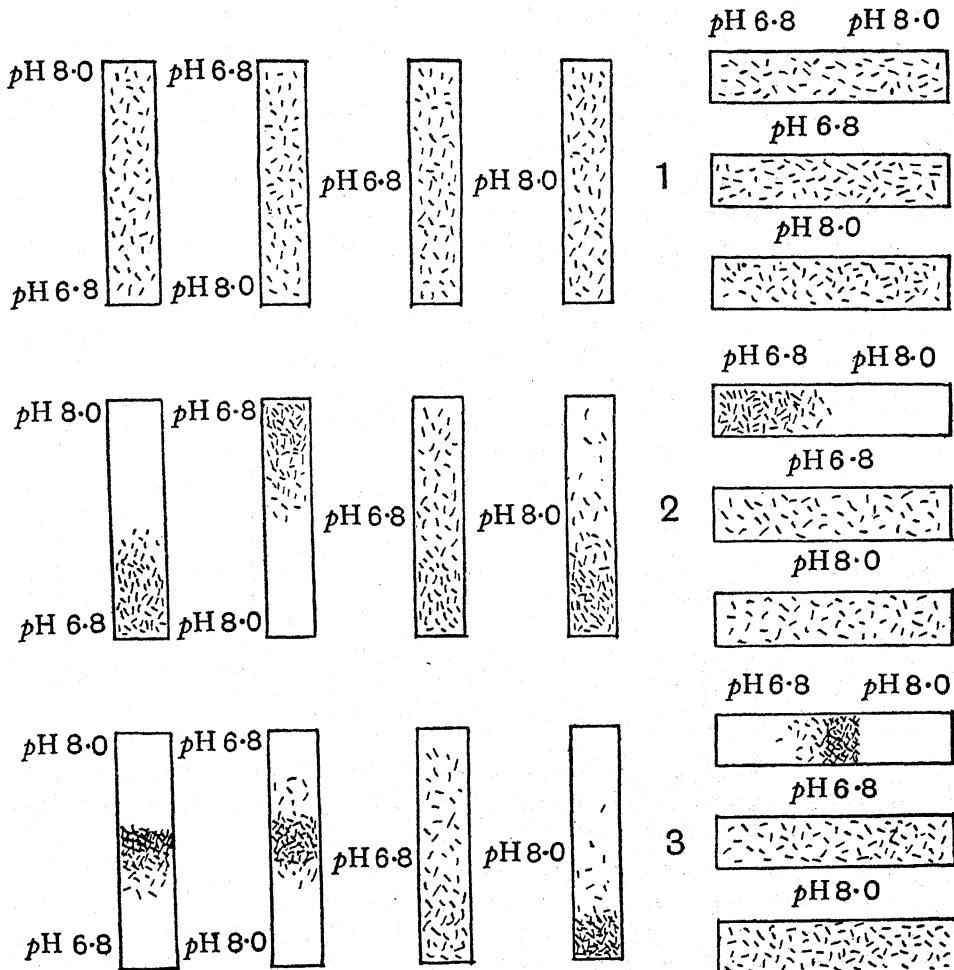


FIG. 3. Diagram representing the appearance of tubes filled with water of different pH and containing *Spirostomum*. (1) at the commencement of the experiment; (2) after 15 minutes; (3) after 60 minutes. The tubes to the left are standing in a vertical position and those to the right are lying in a horizontal position. In the experiment all the tubes were at right angles to the source of light in order to avoid the effects of phototropism.

As I found the wild forms reacted so much more vigorously than the culture forms I used wild forms only for the following experiments:

1. Two samples of the pond water in which the *Spirostomum* were living were taken. One of these had the pH raised to 8.0 by aspirating through it fresh air, while the other sample had the pH lowered to 6.8 by saturating the water with

alveolar air. Spirostomum were then concentrated by centrifuging and added to these samples. The samples together with the Spirostomum were placed in the tubes. Where the two waters of different pH mixed a gradient of hydrogen ion concentration is produced. The first thing that is noticed is an almost instantaneous exodus of the Spirostomum from the pH 8.0 region of the tube into the pH 6.8 region. Fig. 3 shows diagrammatically the distribution of the Spirostomum at the commencement of the experiment, after fifteen minutes and after sixty minutes.

As a result of this experiment we observe:

- (i) That Spirostomum rapidly migrates from water of pH 8.0 into water whose pH is 6.8.
- (ii) That after a time there is a tendency to accumulate in the middle of the tube where the liquids mix and the pH is approximately 7.4.
- (iii) That the attraction of the pH is superior to the attraction of gravity.

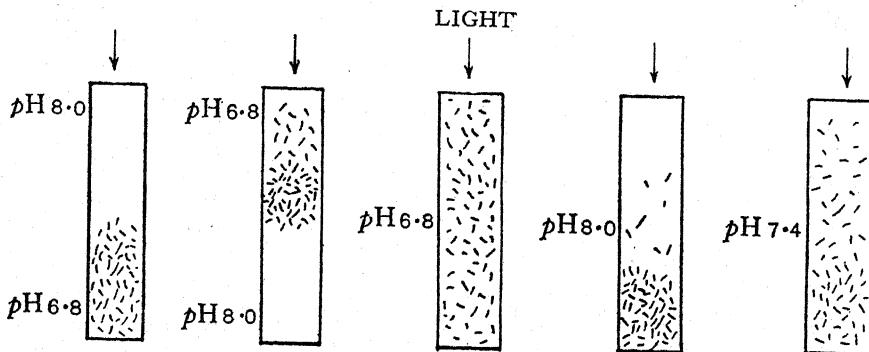


FIG. 4. Diagram representing the appearance of tubes filled with water of different pH and pointing towards the source of light. The tubes are lying in a horizontal position. At the commencement of the experiment the Spirostomum were evenly distributed in each tube.

2. If the tubes, lying horizontal, be pointed towards a source of light then the results shown diagrammatically in Fig. 4 are obtained, after the tubes have been lying for an hour.

From this we see that the phototropism of the Spirostomum varies with the conditions. The animals move out of the alkaline water into the acid whether this movement be towards or away from the light. When the pH is 6.8 they show no reaction at all towards the light, being neither positively nor negatively phototropic. As the water becomes alkaline they show an increasing tendency to become negatively phototropic.

3. In the experiments performed so far the pH at the opposite ends of the tubes has differed considerably. If the difference between the pH of the water at either end be reduced we nevertheless find the same rapid migration into a region of suitable pH . Fig. 5 shows the result of an experiment where the difference in pH at the opposite ends of the tube is no more than pH 0.6. The tubes are lying horizontal and at right angles to the source of light.

From this we see clearly that the Spirostomum moves into the region where the

pH is 7.4 and that this movement may be either up or down the pH gradient. By a further series of very similar experiments I was able to determine that pH 7.4 to 7.45 attracts Spirostomum most. This value corresponds with that which favours most the vital activity and is moreover the same as is found where the Spirostomum are flourishing in cultures or are abundant in ponds or aquaria.

MODIFYING INFLUENCE OF LIGHT ON ATTRACTION OF SPIROSTOMUM
INTO WATER OF CERTAIN pH.

The preceding experiments have all been performed in the light. It is important to mention this as I discovered later that light has a great effect on the preference which Spirostomum shows for water of a particular pH. In fact in darkness this preference is difficult to detect. If a tube, pH 8.0 at one end and pH 6.8 at the other, in which the Spirostomum have migrated into the 6.8 region and left the 8.0 region clear, be placed in darkness, within twenty minutes or half an hour the animals distribute themselves evenly throughout the tube and show no particular preference for any pH. On bringing them into the light again the Spirostomum immediately migrate into the 6.8 region and leave the 8.0 region clear again. From this it is obvious that light has the power of inducing a strong preference for water of a particular pH and that this preference disappears in the absence of light.

A simple way of showing the effect of light and darkness is to take two samples in test-tubes of the culture or pond water in which the Spirostomum are living and to adjust the pH of the samples (by breathing into the tubes and/or shaking) to pH 8.0 and 7.4. In the light the Spirostomum in the sample at pH 8.0 fall to the bottom and remain there, those in the sample at pH 7.4 fall to the bottom but very soon rise again if the test-tube be not disturbed and commence swimming freely in the water. If now without disturbing the test-tubes a cover be placed over them so as to exclude the light for 10-15 minutes it will be seen, on lifting the cover, that the Spirostomum in the water at pH 8.0 have risen off the bottom and are swimming freely in the water, in much the same manner as those in the sample at pH 7.4. The ill effects of the high pH appear to have been negated by placing the animals in the dark*.

In the experiments described above, where the Spirostomum are presented with a choice of waters of different pH, I have used only "wild Spirostomum" and what might be described as "natural buffers," the pond water in which the animals were living having carbon dioxide either added to or abstracted from it in order to produce any required hydrogen ion concentration. Specimens from the aquarium (*S. amboinum*)

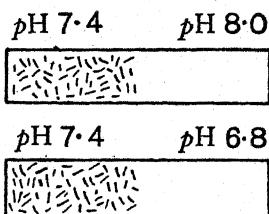


FIG. 5. Diagram representing the appearance of two tubes filled with water of different pH, the differences being less than those shown in Fig. 4. The tubes are lying in a horizontal position and at right angles to the source of light. At the commencement of the experiment, the Spirostomum were evenly distributed in each tube.

* A fuller investigation of this phenomenon, the variation caused by light and darkness in the preference shown for water of a particular pH, and a description of its occurrence in other animals will appear in a later paper.

guum var. *minor*) react nearly as vigorously as the wild forms, but culture forms do not exhibit any such marked preference if natural buffers be used. Culture forms can, however, be usefully employed to demonstrate this same preference for water of a particular *pH* and the absence of this preference in darkness when they are placed in dilute buffer mixtures of phosphates. When the wild Spirostomum are placed in these dilute buffer mixtures they do not behave normally, the animals contract and refuse to move at all. The culture forms seem to be little affected by changing conditions. They are a hardy race quite capable of adapting themselves easily to changes of the environment. The method of culture probably explains this for, when the wild Spirostomum are first placed in the culture tubes, the large majority die and but few survive. These few are those which are able to withstand not only the abnormal conditions of the environment but also the great variations in these conditions which occur more readily and frequently in the laboratory than in the pond. It is reasonable therefore to suppose that a cultured race will be less responsible to small changes in the environment than a purely wild race.

THE BEHAVIOUR OF SPIROSTOMUM IN A NORMAL ENVIRONMENT AND ITS RELATION TO THE *pH* OF THE WATER.

The behaviour of Spirostomum in its normal environment can now be readily explained. During the daytime the animals are found only amongst the leaves at the bottom of the pond where the *pH* of the water is 7.3 to 7.4. The water of the pond itself is *pH* 7.4 to 7.5, the slightly lower *pH* among the leaves is doubtless caused by the decomposition of organic material producing carbon dioxide. In daylight therefore the Spirostomum seek the most acid water, and this is found amongst the leaves. In darkness we should expect from our experiments to find the Spirostomum swimming freely in the water as in fact is the case. During the winter, when the *pH* of the water in the pond remained fairly steady at the values given above (7.3 to 7.4), the Spirostomum behaved in a perfectly regular fashion, hiding among the leaves by day and swimming freely in the water at night. In the spring when the *pH* of the water rose, due to the growth of the plants and the photosynthesis removing carbon dioxide from the water, the behaviour of the Spirostomum became irregular. Finally the Spirostomum disappeared and could not be found anywhere in the pond.

In the collecting pot after the Spirostomum had been brought back to the laboratory they first of all sank to the bottom of the jar. Then for the first night or two they behaved as they did in the pond, becoming planktonic by night and sinking to the bottom and resting there by day. Then they became irregular in their behaviour, often remaining swimming during the day or else remaining on the bottom both by day and night. If the jar were left undisturbed the *pH* of the water in it gradually rose by diffusion of carbon dioxide out of the water until eventually the *pH* reaches 7.7 or 7.8 when the animals aggregate themselves into little clusters at the bottom of the jar. This behaviour of Spirostomum in the collecting jar and the aggregation into clusters were noticed and recorded by Pütter

The explanation of the behaviour of the *Spirostomum* in the collecting jar is probably as follows. When the animals are first collected the shock paralyses them slightly and causes them to fall to the bottom of the jar. The rising in darkness and the falling to the bottom in daylight, which occur for the first day or two after collection, may be attributed to the rhythm acquired in their natural environment. In their natural environment, we must remember, there is a pH gradient, the water at the bottom having a slightly greater concentration of hydrogen ions than the water at the surface. It is apparently the absence of this pH gradient in the collecting jar which eventually causes the irregularities in their behaviour. Even if the pH in the collecting jar be prevented from rising by introducing above the water an atmosphere containing carbon dioxide at the same tension as that dissolved in the water of the pond and then sealing the jar, the behaviour of the *Spirostomum* becomes irregular in just the same way as when the pH is allowed to rise gradually by the jar standing uncovered in the laboratory.

In order to see if this regularity of rising in the darkness and falling to the bottom in the daylight would be maintained if a pH gradient were present, I prepared a long narrow tube, 30 cms. long, standing upright, open to the air at the top and closed at the bottom. At the bottom of this tube I placed a little pond mud which, by its decomposition, produced a regular small supply of carbon dioxide. The water in the tube was coloured by the addition of a few drops of phenol red solution, and after standing for two days the formation of a pH gradient was shown by the indicator. At the bottom the pH was 7.0 to 7.2 and at the surface it was 7.6 to 7.8. On introducing *Spirostomum* into this tube I found that they maintained, in one case for a fortnight, the power of rising by night and falling to the bottom by day. It was difficult to stop the water becoming too acid at the bottom and too alkaline at the surface, but so long as a gentle pH gradient was maintained the animals behaved as they were observed to do in their wild state.

The behaviour of the *Spirostomum* in the aquarium is in many ways similar to that in the collecting jar. Although there was mud on the bottom either the decomposition taking place in it was too little to produce sufficient carbon dioxide or the overlying water was too shallow, for I was unable to detect a pH gradient, and the behaviour of the animals in rising and falling by day and night was distinctly irregular. Nevertheless, the behaviour in this aquarium is of considerable interest in other ways. The aquarium stood in a window facing S.W. and had in it a sufficient quantity of *Spirogyra* to prevent the normal very thorough mixing of the water which occurs in aquaria owing to convection currents. The surface of the water was covered with a bacterial scum which prevented the pH from rising by the hindering diffusion of carbon dioxide from the water into the air. As a result of the *Spirogyra* being present the pH of the water in the aquarium on the side next the window rose to 7.75, while the water on the side away from the window was pH 7.5. No *Spirostomum* were to be found where the water was pH 7.75, all being congregated on the side away from the light where the pH was 7.5. On sunny days the photosynthetic activity of the *Spirogyra* was increased and slightly more carbon dioxide was removed from the water. On these days the pH of the water on the

side near the window would rise to 7.85 or 7.9 while the *pH* on the side away from the window rose to 7.55. This slight rise in the *pH* of the water was responsible for the Spirostomum aggregating together into large clusters on the bottom and on the Spirogyra itself. Some of these clusters were 1 cm. in diameter and were composed of a solid mass of Spirostomum. On sunny days the Spirostomum were in clusters and on dull days they were swimming freely in the water. On all occasions when I found the Spirostomum thus swimming freely in the water the *pH* never exceeded 7.5 and was usually 0.1 to 0.2 *pH* less than this. As the days lengthened the light increased and the Spirogyra by its photosynthetic activity never allowed the *pH* to fall below 7.6. The Spirostomum were always in clusters. These clusters became smaller and smaller until finally the Spirostomum disappeared from the aquarium.

The clusters on the bottom appeared to burrow into the mud, and I suggest that in this manner some few animals are enabled to survive the period when the aquarium water is raised above *pH* 7.8. They obviously cannot survive for very long in water of this *pH* as my experiments have shown unless they can encyst (and there is no evidence to show that Spirostomum ever does this) or they can find some place where the *pH* does not rise to lethal values. The only place where such a *pH* is found is in the mud where decomposition is taking place.

The clustering of Spirostomum is evidently a function of the hydrogen ion concentration, the critical value being *pH* 7.5 to 7.6. It is a remarkable phenomenon and was observed by Pütter. It can be produced quite easily in wild forms by keeping them in water adjusted to the critical *pH* either by the abstraction or addition of carbon dioxide, when aggregation in clusters will occur in from 24 to 48 hours. In culture forms I was unable to produce this aggregation either in natural or artificial buffers.

THE RELATION OF *pH* TO CONJUGATION.

At the beginning of May 1923 Miss Bishop found a number of tubes of her cultures of Spirostomum contained forms that were conjugating. I tested the *pH* of these cultures in which the conjugating forms occurred and also the *pH* of tubes of cultures in which no conjugation was to be observed. I could detect no differences between the *pH* of these tubes such as would lead one to suppose that conjugation was connected with the *pH*. Moreover the *pH* of the tubes containing conjugating forms was, on the average, the same as the *pH* found during the winter when none of the culture tubes showed any signs of conjugating forms.

TEMPERATURE.

All the above experiments and observations were made at room temperature, which varied from 9.0 to 15° Centigrade. No attempt was made to control the temperature during the experiments. The temperature of the pond from which the wild Spirostomum were obtained often differed by as much as 10° Centigrade from that of the room in which the experiments were performed. There is, however,

no possibility of a temperature gradient having occurred under the experimental conditions for it was necessary for the temperature of the solution to be uniform throughout in order to prevent the solutions of different pH from mixing completely.

SUMMARY.

1. In a pond in which Spirostomum occurred in vast quantities the pH of the water was 7.4, that of an aquarium in which Spirostomum were present in large numbers was 7.5, and that of the water containing flourishing cultures of Spirostomum was 7.4 to 7.6.

2. The Spirostomum showed the greatest activity in solutions of pH 7.4. Below this value the activity is reduced, but down to pH 6.0 at any rate the effect is not lethal. In solutions above pH 7.6 to 7.8 the Spirostomum are killed within a few hours if the pH greatly exceeds these values, and in a longer time if they are barely exceeded. In dying as result of the alkalinity of the water being too great the animals assumed a characteristic pear-shaped malformation.

3. Pütter (1903) supposed that the death of Spirostomum which occurred when the animals were placed in shallow vessels exposed to the atmosphere was due to oxygen poisoning. This is shown not to be the case but to be due to the pH rising above the lethal value.

4. Spirostomum, when placed in tubes in which the pH of the water at one end is 8.0 and 6.8 at the other, are found to migrate rapidly into the water of pH 6.8 and then finally settle in the middle of the tube where the water is pH 7.4. This preference for water of a particular pH, which is stronger in its effects than those of light and gravity, disappears in the darkness.

5. The normal behaviour of Spirostomum in the aquarium, in the pond and in the collecting jars may be explained to a large extent by the preference for water of a particular pH and by the presence or absence of a vertical pH gradient.

6. No evidence was found to connect an epidemic of conjugation with the pH of the water.

7. The temperature at which the experiments were performed or the observations made varied from 9 to 15° Centigrade, but the temperature was uniform throughout any tube or jar at the time it was observed. There was no temperature gradient.

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ON CHLOROCRUORIN. I

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(With Eight Text-figures.)

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I. INTRODUCTION.

CHLOROCRUORIN is a green pigment dissolved in the blood plasma of certain polychaetes, namely the Chlorhaemidae and the Sabelliformia. It was first observed by Milne-Edwards (1838) and studied by Ray Lankester (1867, 1870), Krukenberg (1882), MacMunn (1885, 1889) and Griffiths (1892). These authors showed that the pigment exists in two forms, oxidised and reduced. Oxychlorocruorin has two absorption bands, the limits of which were given as $618-593\text{ }\mu\mu$ and $576-554\text{ }\mu\mu$. This was reduced by ammonium sulphide to a form having one band between 625 and 596. Lankester considered chlorocruorin to be a respiratory pigment because the reduced form re-assumes the bands of oxychlorocruorin on being shaken with air. Further, Lankester showed that the pigment is related to haemoglobin since he obtained Stokes' reduced haematin from it. Griffiths, too, obtained haematin.

The present paper gives an account of the first stages of an investigation of chlorocruorin carried out this summer at the Roscoff Marine Biological Station, to the Director of which I am indebted for an abundant supply of material. My particular thanks are due to Mr J. Barcroft, F.R.S., for instruction in methods. It was at his suggestion that I undertook an investigation of invertebrate respiratory pigments. I am extremely indebted to Prof. Fred Vlès for most valuable advice and for permission to use his spectrophotometer.

The blood studied was obtained from *Spirographis Spellanzanii*, while observations on the circulation were made on *Sabella pavonina*, *Branchiomma vesiculosum*, *Dasychone bombyx* and *Amphiglena mediterranea*. It is difficult to obtain pure blood in quantity even from such a relatively large worm as *Spirographis*. The greater part of the blood is contained in the peri-intestinal sinus and in extraction is easily diluted with coelomic fluid or contaminated with gut contents. After a number of trials a technique was developed by which pure blood could be extracted with a capillary pipette from the plexus around the oesophagus. As much as 1 c.c. of blood can be obtained in an hour from one individual.

The colour of the blood is red when seen in the blood vessels by reflected light. By transparency under the microscope the blood is green in small vessels but red when two are superposed. In a glass vessel the blood is red when concentrated, green diluted.

2. CHLOROCRUORIN AS A RESPIRATORY PIGMENT.

The first step was to discover whether chlorocruorin is a respiratory pigment in the sense of absorbing a greater amount of oxygen than does water and giving up this oxygen to tissues. Like a number of other invertebrate pigments chlorocruorin has been assumed to be respiratory on the ground that it can be reduced by reducing agents and re-oxidised in the air. This, however, in no way demonstrates that reduction can take place in the animal body.

I soon found that chlorocruorin is indeed a respiratory pigment, for it can be reduced (1) by a vacuum, and (2) by living tissues, and subsequently be re-oxidised by the air. The changes were followed with a Zeiss microspectroscope. The oxy-chlorocruorin in a drop of blood was reduced by a vacuum in a special chamber on the microscope stage, made to the design of my friend Dr Keilin, who has given me much help in technique. The vacuum was produced by a mercury pump. The two bands of oxychlorocruorin changed to the single band of the reduced form. On readmitting air the bands of oxychlorocruorin appeared again instantaneously.

Reduction of the pigment by living tissues was brought about under a coverslip sealed with vaseline. A fragment of muscle surrounded by diluted blood reduced the oxychlorocruorin of the blood. In another similar experiment the oxychlorocruorin was reduced while still inside a blood vessel by a piece of muscle lying near the vessel. The blood sacs closed at one end which project into the coelom were used. In this case the reduction took half an hour. On raising the coverslip the oxychlorocruorin bands reappeared instantaneously in the blood vessel.

Under the microscope the colours of oxychlorocruorin and reduced chlorocruorin could not be distinguished from one another.

It was next found that the blood gives up a gas on the addition of potassium ferricyanide. The amount of gas liberated from 1 c.c. of blood was determined with a Barcroft microdifferential apparatus. Assuming that the gas is oxygen and that the whole amount which would be given up to a vacuum is liberated by the ferricyanide, as in the case of oxyhaemoglobin, the determinations made give the total oxygen capacity of the blood. Extractions will shortly be made with a vacuum pump which will decide whether or not these assumptions are justified*.

Determinations were made first with my own blood and then with the blood of *Spirographis*. Only relative not absolute values of gas liberated can at present be given as the constant (*k*) of the apparatus has not yet been determined. Three estimations with my blood gave 41.0 k., 40.6 k. and 41.3 k. cubic mm. O₂ from 100 cubic mm. blood, after correcting for temperature, pressure, and delivery of pipette. The average of these values is 41.0 k. For eight determinations of *Spiro-*

* Winterstein (1909), using a mercury pump, determined the total oxygen capacity of body fluids containing haemocyanin, haemerythrin, and echinochrome, but he was unable to study chlorocruorin owing to the small quantities of blood obtainable.

graphis blood the following figures were obtained: 14.5 k., 14.0 k., 15.2 k., 14.1 k., 12.9 k., 14.6 k., 13.2 k., 12.6 k. The average is 13.6 k. volume per cent. The total oxygen capacity of *Spirographis* blood is thus $\frac{13.6 \text{ k.}}{41.0 \text{ k.}} = .332$ or one-third that of my own blood.

Assuming that 100 c.c. of my blood contain 18.5 c.c. oxygen, 100 c.c. of *Spirographis* blood would contain 6.17 c.c. O₂, or 10.3 times the quantity dissolved in sea-water. This is equal to the highest value for the oxygen capacity of *Arenicola* blood deduced from the figures of haemoglobin content given by Barcroft. The latter states (1924, p. 29) that Arenicolan blood contains from $\frac{1}{3}$ to $\frac{1}{2}$ the quantity of haemoglobin which would be in the same volume of human blood. The blood of another polychaete with haemoglobin, *Glycera*, contains only 2.5-3.0 vols. per cent. O₂ (Winterstein, 1909).

Although chlorocruorin is a respiratory pigment in the sense defined above, it is as yet undecided what rôle it plays in the economy of the worm. The pigment does not appear to serve for transporting oxygen from the surface of the body to the internal organs for the blood circulation is very incomplete.

Most of the blood is contained in a peri-intestinal sinus. In *Spirographis*, in addition to this, a considerable amount of blood is contained in numerous finger-like sacs, closed at one end, which project like internal gills from the body wall into the coelom. Further, a quantity of blood is found in vessels running in the crown of branchiae. A good description of the anatomy of the vascular system of *Spirographis* is given by Claparède (1873), except that little mention is made of the "internal gills."

Peristaltic contractions from behind forwards pass along the peri-intestinal sinus, but these contractions can hardly move the blood in the "internal gills" of *Spirographis*. The walls of these sacs are apparently non-contractile. The contractions of the peri-intestinal sinus can easily be observed in small transparent worms such as *Amphiglena* or young *Branchiomma*, .5 cm. long. Here the sinus is enormous relative to the volume of other vessels. The contractions in *Amphiglena* occur every 5 seconds. It can clearly be seen that the blood circulates very little. The blood is squashed forwards out of a given region of the sinus by complete elimination of the lumen. When the wall of the sinus is again relaxed most of the blood which had been forced forward flows back again to its old position.

In the branchiae there is a single blood vessel along each main branch with a single offshoot ending blindly at the distal termination of each side pinnule. Here again the blood does not circulate, contrary to the statement of Claparède, who says (1873, p. 81) that the blood is moved from the base to the tips of the branchiae by centrifugal contractions of the vessels and then moved back again to the base by centripetal contractions. Such is not the case. There are rhythmic peristaltic contractions but they are always from the tips to the base of the branchiae. These contractions can cause no circulation but merely an alternate to and fro movement of the blood in each part of the vessel.

The wave of contraction is rapid, occupying in *Dasychone* (total length of worm

1 cm.) a fraction of a second from end to end of the gill. There is a slight dilatation immediately before each contraction. In *Sabellula* the waves can be seen to be synchronous in the main gill branches. Moreover, the wave is timed to start from the tip of each pinnule so as to arrive at the base of the pinnule exactly at the moment at which the wave passing down the main branch reaches that point. The rhythm varies in different species and in one and the same species under different conditions. In *Spirographis* (15 cm. long) with gills expanded in aerated water at 17° the period (a) from the commencement of dilatation to the commencement of the ensuing contraction is on the average 8 seconds, while the period (b) from the commencement of contraction to its end is 7 seconds. In *Dasychone* (1 cm. long) (a) is 6 seconds, and (b) 1.5 seconds.

Thus there appears to be no regular blood circulation from the branchiae or body surface to the internal organs such as would be necessary if chlorocruorin actively transported oxygen. In any case it has been shown by Bounhiol (1902) that the branchiae of Sabellids are not specialised respiratory organs. The respiration of *Spirographis* after amputation of the branchiae decreased only to three-quarters of its initial value. In other specimens when the body was covered with vaseline the respiration, now through the branchiae alone, decreased to a quarter of its former amount. It might have been supposed nevertheless that respiration through the general body surface would be restricted in natural conditions by the presence of the tube, so that the principal gas exchange would take place through the branchiae. This is not so, however, for animals in their tubes and out of their tubes respired at the same rate (Bounhiol, p. 80).

The chlorocruorin again might act as a reservoir of oxygen, to be used when the oxygen tension in the water fell so low that an insufficient amount was in physical solution to satisfy the requirements of the animal. But such an event cannot normally occur in the life of *Spirographis*. At low tide the tubes are either projecting from the sand in shallow, *i.e.* well oxygenated water, or more rarely exposed to the air. Under the latter conditions Bounhiol has shown (1902, p. 73) that annelids do not suffer from oxygen want but respire as actively as when submerged.

Lastly, it may be that chlorocruorin has no function at all, although it may have an effect. Bounhiol (1902, p. 40) showed that polychaetes with haemoglobin have a more active gas exchange than those without haemoglobin, but it does not follow that this more active respiration is essential to their well-being. Indeed, *Spirographis* can be caused to respire less actively (partial asphyxia) by reducing the oxygen supply (Bounhiol, 1902, p. 102) "sans inconveniencé." It will be shown below that chlorocruorin like haemoglobin forms a compound with CO which cannot be reduced. Experiments are being made with the object of putting the chlorocruorin out of action by this means inside the body of the worm.

3. AFFINITIES OF CHLOROCRUORIN.

The investigation of the chemical relationship of chlorocruorin to be described below has been chiefly spectroscopic. As in the preceding section the results so far obtained are preliminary.

It is at once obvious that the two absorption bands of oxychlorocruorin and one band of reduced chlorocruorin resemble the bands of oxyhaemoglobin and haemoglobin shifted towards the red end of the spectrum. The resemblance does not end here. From chlorocruorin I have obtained a series of derivatives corresponding to the haematin and haematoporphyrins. The substance corresponding to haemochromogen has similar bands to the latter, again shifted towards the red but to a lesser distance than in the case of oxychlorocruorin and chlorocruorin, compared with oxyhaemoglobin and haemoglobin. The porphyrin, however, is either identical or very nearly so with haematoporphyrin.

Here, then, is apparently a unique case of a parallel evolution of a substance resembling haemoglobin. The start is from the same basis, the porphyrin. When iron is added there is a divergence, when the protein is super-added there is a further divergence. Support is given to this hypothesis of a close relationship between chlorocruorin and haemoglobin by the fact that chlorocruorin and the chromogen in combination with CO and with CN behave spectroscopically in the same way as do haemoglobin and haemochromogen. Further, chlorocruorin like haemoglobin acts as a peroxidase (Lankester, 1870; Prenant, 1921; Romieu, 1923).

Yet the spectra of the derivatives of chlorocruorin corresponding to the haematin and to methaemoglobin have peculiarities of their own, and a detailed study of the spectra of oxychlorocruorin and chlorocruorin shows considerable divergences from the plan of the oxyhaemoglobin and haemoglobin spectra. A true test of the hypothesis will only be forthcoming when the spectrophotometric curves of each of the products have been made, including those in the ultra-violet. This work is now in progress. Below are given the details of the qualitative study of all the spectra, together with spectrophotometric curves of oxychlorocruorin and chlorocruorin.

The terminology of the derivatives of chlorocruorin presents a difficulty. For uniformity names should be given resembling the names of the corresponding haemoglobin derivatives. Had chlorocruorin been called "cruoroglobin" this would have been simple, but the name being what it is the designations of the derivatives are necessarily cumbersome. The following list gives the names decided upon for those derivatives which have been prepared. The corresponding haemoglobin derivatives are indicated:

Oxyhaemoglobin	Oxychlorocruorin
CO-haemoglobin	CO-chlorocruorin
Haemoglobin	Chlorocruorin
Methaemoglobin	Metachlorocruorin
Haematin	Chlorocruorohaematin
Haemochromogen	Chlorocruorochromogen
CN-haemochromogen	CN-chlorocruorochromogen
Haemin	Chlorocruorohaemin
Haematoporphyrin	Chlorocruoroporphyrin

(a) OXYCHLOROCRUORIN.

The absorption spectrum of O_2Ch (abbreviation for oxychlorocruorin) shows a dark and a light absorption band (Fig. 1, I). On the analogy of the oxyhaemoglobin bands they will be called α and β . The α band extends from $\lambda_{\mu\mu}$ 619–591, 616–593 and 612–599 in three increasing dilutions of blood. The position of the β band is 569–551 and 565–551 in the more concentrated and the medium dilutions of blood. In the highest dilution examined it could just be seen at 560. The optical axes of the bands, *i.e.* the position of greatest absorption of light seen, are for α at 604, for β at 560. In relatively concentrated blood which has been clarified by the centrifuge a third very light band is visible at 518.

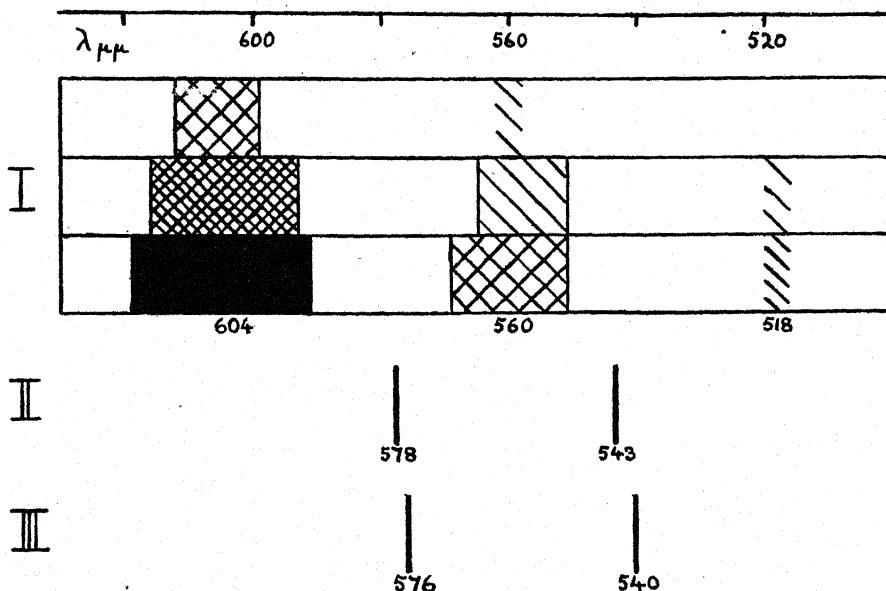


FIG. 1. Absorption spectra. I: Oxychlorocruorin in three different concentrations. The numbers give the optical axes of the bands. II and III: Spectrophotometric axes of the bands of oxyhaemoglobin of the horse (II) and of *Arenicola* (III), from Vlès, 1923, p. 7.

The two main bands resemble those of oxyhaemoglobin shifted far towards the red. Vlès (1923) has shown that the invertebrate haemoglobins differ from that of mammals and differ among themselves by slightly varying situations of the axes of the bands. The positions of the axes are shown in Fig. 1 (II and III) for mammalian and Arenicolan O_2Hb . The former differs from the latter in that its bands are slightly shifted towards the red. The bands of O_2Ch are shifted very much further into the red.

The question is now being investigated as to whether there is one or more than one chlorocruorin. The details given here all apply to *Spirographis*.

Fig. 2 (I) gives the spectrophotometric curve of O_2Ch . The axes of the bands are at 609 and 517. These numbers are not identical with those given above.

which were obtained from a direct inspection of the spectrum. Such divergences are normal and are caused by the fact that the eye is deceived in a direct inspection by differential sensibility of the retina to contrasts of colour and of light intensity.

The curve shows that the summit of the α band is much higher than that of the β . In mammalian O_2Hb , too, the summit is higher but the difference is much less marked (Vlès, 1921, Fig. 1), while in other vertebrates (Ostermann, 1907 and Gallerani, 1912), and in *Arenicola*, *Marphysa*, *Lumbricus* and *Chironomus* (Vlès, 1923, Fig. 3 and p. 22) β is the higher. The third summit at 517 in O_2Ch has no parallel in O_2Hb . It remains to be seen, however, from the examination of a number of samples under different conditions whether this band remains constant or whether it is due to an impurity.

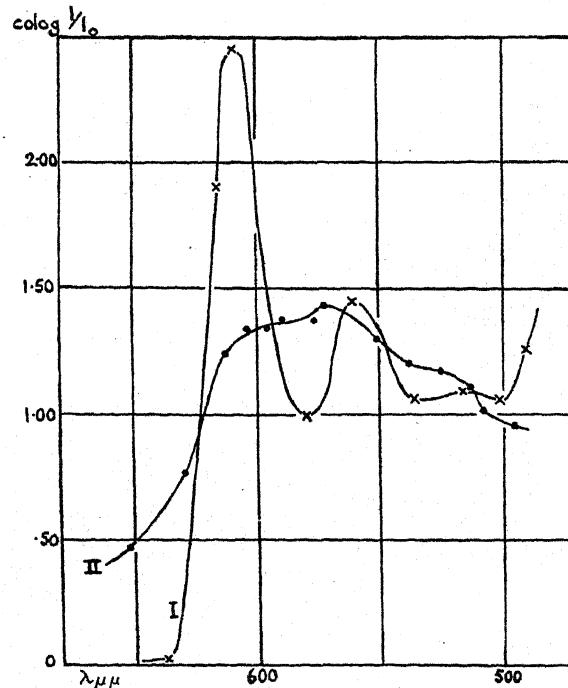


FIG. 2. Spectrophotometric curves of (I) oxychlorocruorin and (II) chlorocruorin. The molecular concentrations are the same for the two curves. After the oxychlorocruorin had been determined it was reduced in the trough with $Na_2CO_3 + Na_2S_2O_4$.

(b) REDUCED CHLOROCRUORIN.

The colour difference to the naked eye between O_2Ch and Ch (the abbreviation for reduced chlorocruorin) is very slight, but Ch (reduced with hydrosulphite) is of a rather more yellowish-green than O_2Ch . The spectrum of Ch is shown in Fig. 3 (I and II). There is a broad band divided into several parts. It is much less intense than the α band of O_2Ch . The spectrum is different according to the reducing agent employed. With sodium hydrosulphite in alkaline solution (Fig. 3, I) the band extends, in a medium dilution of blood, from 623-563. The darkest part

is between 588 and 563 with the axis at 574. There is a second dark region, not so pronounced, from 623-599 with an axis at 610. Between these two there is a lighter region from 599-588. In addition, there is a very feeble band at 525. Reduced instead with ammonium sulphide the main band extends from 619-573 and has a single axis at 589, while there is a feeble band about 518.

The spectrophotometric curve of Ch reduced by hydrosulphite is shown in Fig. 2 (II). It gives quite a different interpretation to the spectrum from that suggested by direct inspection (cf. Fig. 3, I). There is a single very broad band

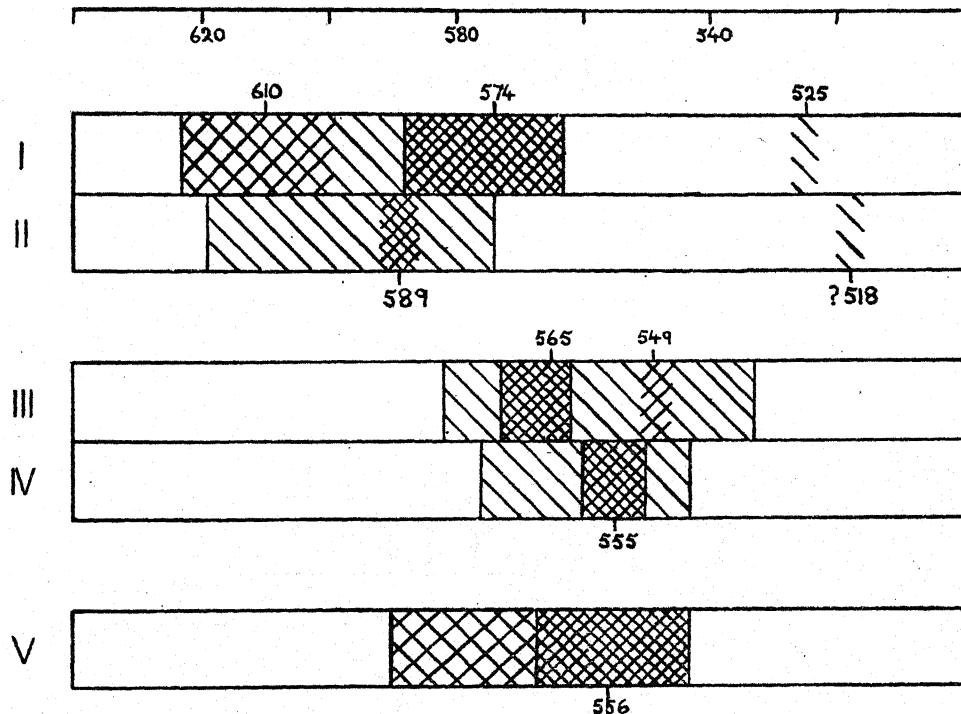


FIG. 3. Absorption spectra. I: Chlorocruorin, reduced by $\text{Na}_2\text{CO}_3 + \text{Na}_2\text{S}_2\text{O}_4$. II: Chlorocruorin, reduced by Am_2S . III: Arenicolan haemoglobin, reduced by $\text{Na}_2\text{CO}_3 + \text{Na}_2\text{S}_2\text{O}_4$. IV: Arenicolan haemoglobin, reduced by Am_2S . V: Mammalian haemoglobin. III, IV and V from Vlès, 1923, Fig. 5. The numbers indicate the axes of the bands, in I and II optical axes, in III, IV and V spectrophotometric axes from Vlès, 1923, p. 17 and 1921, p. 12.

with a summit at 573 and a buttress on either side. That on the left is the bigger and represents the left-hand reinforcement of the main band in Fig. 3 (I), that on the right is less marked and is the small band, apparently independent, on the right in Fig. 3 (I). It is evident that there are in reality no valleys between the summit and the buttresses, contrary to what is suggested by Fig. 3 (I).

Vlès has shown (1923, p. 17) that Arenicolan Hb has a spectrum which varies with the reducing agent employed in its preparation (Fig. 3, III and IV). This is in direct contrast to the Hb of mammals which is the same (Fig. 3, V) whatever reducing agent is used, but it resembles the behaviour of Ch (Fig. 3, I and II).

At first sight the spectrum of the hydrosulphite Ch resembles in appearance

that of the hydrosulphite Arenicolan Hb (cf. Fig. 3, I and III). A comparison of the spectrophotometric curves, however, shows the essential difference (cf. Fig. 2, II with Vlès, 1923, Fig. 4). The Arenicolan Hb has two distinct summits. Contrast also with this the single summit of the curve of mammalian Hb (Vlès, 1921, Fig. 3).

Resuming, O_2 Ch has two main bands much further into the red than those of O_2 Hb. The mammalian O_2 Hb bands, however, are slightly more towards the red than the Arenicolan. The α band of O_2 Ch is much higher than the β . In mammalian O_2 Hb α is slightly higher, in non-mammalian slightly lower than β . O_2 Ch has a third band at 517 which is not represented in O_2 Hb.

Reduced Ch has a broad band much further into the red than Stokes' band of Hb. Ch resembles invertebrate and differs from mammalian Hb in that the spectrum varies with the reducing agent. Ch has one summit with a buttress on either side, mammalian Hb has a simple summit while Arenicolan Hb has two summits.

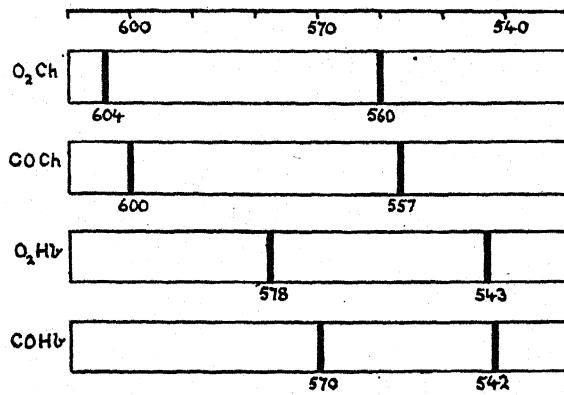


FIG. 4. Axes of absorption bands. O_2 Ch: Oxychlorocruorin. COCh: Carboxy-chlorocruorin. O_2 Hb: Mammalian oxyhaemoglobin (spectrophotometric measurements, Vlès, 1921, p. 7). COHb: Mammalian carboxyhaemoglobin (classical figures).

(c) CO-CHLOROCRUORIN.

As in the case of O_2 Hb, CO moves the bands of O_2 Ch towards the blue. The axes of the α and β COCh bands, determined by direct inspection of the whole spectrum, are at 600 and 557, whereas, as stated above, the axes of the O_2 Ch bands are at 604 and 560. Fig. 4 gives a comparison of COCh with COHb. It is impossible to reduce COCh with alkaline hydrosulphite.

Barcroft has shown (1924) that there is a quantitative relation between the affinity of haemoglobin for gas and the situation of the absorption bands. A comparison was made of the affinities for O_2 and for CO of human and Arenicolan haemoglobin. The affinity was measured by taking the reciprocal of the pressure at which the pigment is 50 per cent. saturated with the gas. It was found that the logarithm of this reciprocal bears a linear relation to the position of the α band. The further the band is situated towards the blue the greater is the gas affinity. Now the differences between the positions of the α bands of O_2 Hb and COHb in various

animals are of a very much smaller order of magnitude than the differences between these and the positions of the α bands of O_2Ch and $COCh$. If Barcroft's law can be extended to substances so far removed from haemoglobin as chlorocruorin the affinity of the latter for gases must be much less than that of haemoglobin. The dissociation curves of chlorocruorin now being prepared will decide this question.

(d) METACHLOROCRUORIN.

The derivative which should be comparable with methaemoglobin was prepared by adding potassium ferricyanide crystals to *Spirographis* blood. The colour of the product was brown. Its spectrum showed two weak bands, the first 612-590 with its axis at 604, the second 571-561 with its axis at 569. This does not resemble the methaemoglobin spectrum. The addition of solid Na_2CO_3 with the object of preparing the alkaline form produced no change at all in the spectrum. Here, then, is a second difference from methaemoglobin. The solution of solid potassium bichromate in the liquid again caused no alteration in the bands.

Vlès has shown (1923, p. 19) that the Met-hb of *Arenicola* has not the typical Met-hb bands, but that the acid and alkaline Met-hb resemble acid and alkaline haematins. The Arenicolan haemoglobin molecule then is differently constituted to that of vertebrates. Metachlorocruorin, however, does not resemble chlorocruorohaematin, but it differs in another way from methaemoglobin. This is seen both from the nature of the bands and from the apparent indifference to pH .

(e) CHLOROCRUOROHAEMATIN.

If chlorocruorin is constructed on the same plan as haemoglobin the addition of glacial acetic acid to *Spirographis* blood should produce the substance corresponding to haematin. The spectrum of the product shows a single band at 583. This is the average of the axis in three separate preparations, which had the values 582, 586, 581. Such variation would be normal in a haematin since the exact position of the bands depends on the constitution of the medium. The single band does not resemble an acid but an alkaline haematin (see Fig. 5). Solid $NaOH$ was next added to the liquid. Again a single band at 580 appeared. This is the average value from four separate specimens, the axes of which were at 579, 583, 578 and 581. It looks, then, as if the alkaline chlorocruorohaematin were present not only in $NaOH$ but also in acetic acid. Possibly the pH of acetic acid is not low enough to allow the acid form to appear.

(f) CHLOROCRUOROCHROMOGEN.

Whereas metachlorocruorin and chlorocruorohaematin have a different spectroscopic aspect from methaemoglobin and haematin, the derivative corresponding to haemochromogen resembles the latter. The preparation was made by treating *Spirographis* blood with acetic acid to form chlorocruorohaematin, dissolving solid $NaOH$ in this to give an alkaline reaction and then reducing the pigment with hydrosulphite. The resulting solution is brownish-red with a green tinge.

The spectroscope shows a strong band from 596 to 553 with a more intense middle portion from 573-564, having its axis at 569. In addition there is a lighter band between 541 and 524 with its axis at 533 (Fig. 6, II). It is at once obvious that this resembles the spectrum of haemochromogen (Fig. 6, IV) with the bands shifted towards the red. The shift is, however, less in extent than in the case of $O_2\text{Ch}$ compared with $O_2\text{Hb}$ (cf. Fig. 1 with Fig. 6).

The fact that chlorocruorochromogen, while resembling haemochromogen, is by no means identical with the latter, gains in interest from the fact that the haemochromogens prepared from the slightly differing haemoglobins of various animals are themselves practically identical (Vlès, 1923, p. 20).

When an excess of hydrosulphite is used in the preparation of chlorocruorochromogen the spectrum shown in Fig. 6, II, is obtained at once, but when a small quantity only of hydrosulphite is employed the bands have at first the aspect shown in Fig. 6, I. This slowly changes into the form of Fig. 6, II.

In some preparations, but not in all, an additional light band appeared at 619. When absent it can be called into being by agitating the solution with air.

The close relationship of chlorocruorochromogen and haemochromogen is emphasised by their behaviour on the addition of KCN. The bands become less intense and move towards the red (Fig. 6, III and V). The reaction is gradual, not immediate.

(g) TEICHMANN'S CRYSTALS.

Spirographis blood dried on a microscope slide and treated with the usual procedure for preparing haemin gives crystals which resemble the latter. Until a crystallographic examination has been made it is impossible to say whether or not they are identical.

(h) CHLOROCRUOROPORPHYRIN.

The derivative of chlorocruorin prepared in the same way as haematoporphyrin (Hphy) is prepared from haemoglobin is here called chlorocruoroporphyrin (Chphy). The general aspect of the spectrum of Chphy closely resembles that of Hphy and the situations of the axes of the bands are very similar. Since the exact position of the bands of Hphy varies with the nature of the medium it was essential to compare Chphy with Hphy prepared by an identical procedure. Even employing the same procedure the axes differed slightly in wave length on each occasion. The results of the series of preparations are shown in Fig. 7 for the acid porphyrins and Fig. 8 for the alkaline porphyrins. The resemblance between Chphy and Hphy is seen to be close, but whether or not there is identity can only be decided when the spectrophotometric curves have been completed and crystallisations made.

The mode of preparation was as follows. The blood (whether of *Spirographis* or my own) was dried on a water bath and then dissolved in warm pure H_2SO_4 . The solution of Chphy is deep red-brown. The bands are less intense than in Hphy. To prepare the alkaline form the acid Chphy or Hphy was diluted with water and then solid NaOH was dissolved in the liquid, kept cool under a tap. The alkaline

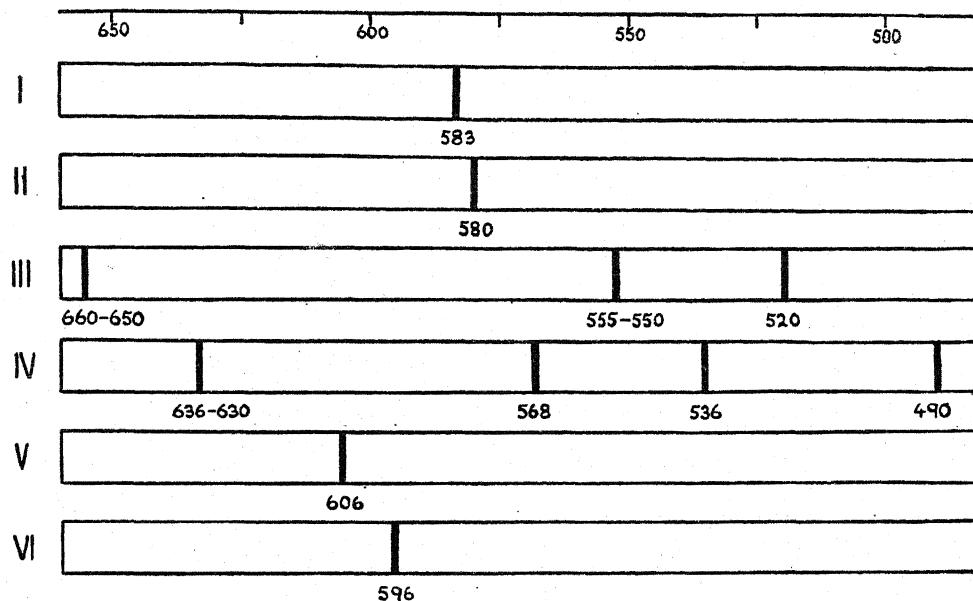


FIG. 5. Axes of absorption bands. I: Chlorocruorohaematin, acetic acid. II: Chlorocruorohaematin, NaOH. III: Haematin, very acid. IV: Haematin, slightly acid. V: Haematin, faintly alkaline, VI: Haematin, very alkaline. III, IV, V and VI are unpublished figures of Prof. Vlès.

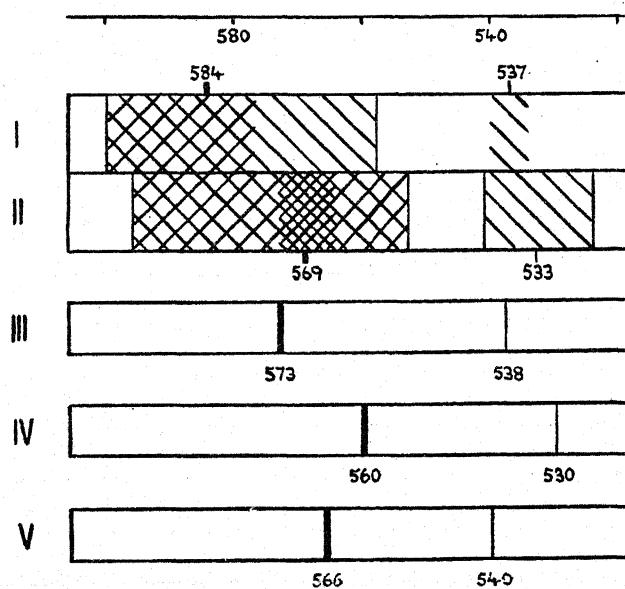


FIG. 6. I: Initial stage of chlorocruorochromogen. II: Final stage of chlorocruorochromogen. III: CN-chlorocruorochromogen. IV: Haemochromogen. V: CN-Haemochromogen (Vlès, 1920, p. 237).

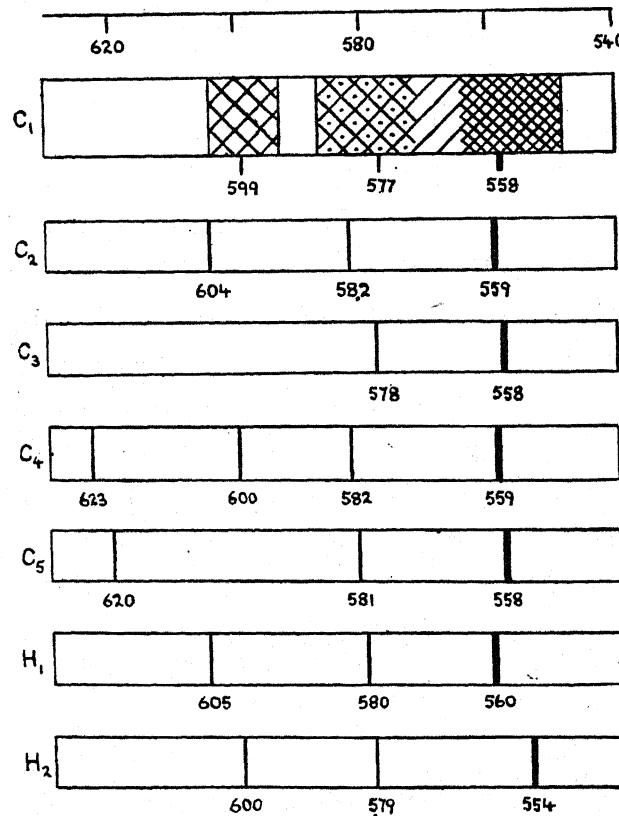


FIG. 7. C₁–C₅: five specimens of acid chlorocruoroporphyrin. H₁, H₂: two specimens of acid haematoporphyrin.

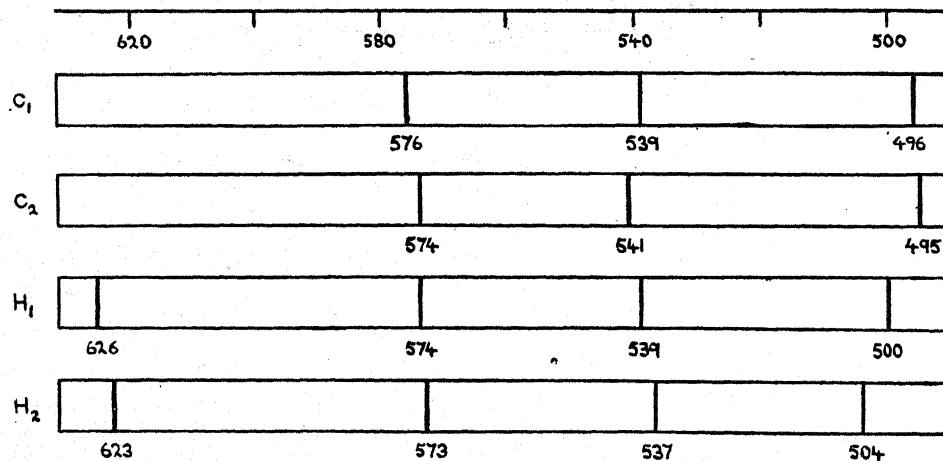


FIG. 8. C₁, C₂: two specimens of alkaline chlorocruoroporphyrin. H₁, H₂: two specimens of alkaline haematoporphyrin.

Chphy was a light yellow solution. The bands here were extremely faint. As in Hphy the band about 500 was the strongest. In alkaline Hphy the band at 623-626 is the weakest. This is presumably the reason for its absence in the alkaline Chphy.

Hphy has a brilliant red fluorescence due to the ultra-violet (Dhéhé et Sobolewski, 1911). Chphy exhibits the same phenomenon. The procedure employed to demonstrate this was as follows. The acid porphyrin was dropped into alcohol. The turbid liquid was cleared with HCl. Sunlight was then concentrated on to the glass tube with a hand lens.

4. SUMMARY.

1. Chlorocruorin, the red-green pigment dissolved in the blood of certain polychaetes, exists in an oxidised and a reduced form, which have different absorption spectra. Chlorocruorin is a respiratory pigment in that the oxidised form can be reduced (*a*) by a vacuum, and (*b*) by living tissue, and then reoxidised by air.

2. Blood of *Spirographis*, containing chlorocruorin, gives off on the addition of potassium ferricyanide one-third of the volume of gas given off by my own blood.

3. The absorption bands of oxychlorocruorin and reduced chlorocruorin resemble those of oxy- and reduced haemoglobin shifted towards the red end of the spectrum.

4. The oxychlorocruorin spectrum differs from that of oxyhaemoglobin in the relative heights of the α and β bands and in the presence of a third much smaller band.

5. Reduced chlorocruorin resembles the reduced haemoglobin of *Arenicola* in that the spectrum varies with the reducing agent employed.

6. The band of reduced chlorocruorin has a summit with a buttress on either side. Thus it differs from the simple summit of the mammalian and the double apex of the Arenicolan haemoglobin band.

7. The spectra of metachlorocruorin and chlorocruorohaematin differ considerably from those of methaemoglobin and haematin.

8. Chlorocruorochromogen closely resembles haemochromogen spectroscopically. The bands of the former are further towards the red than those of the latter, but the shift is less than in the case of oxy- and reduced chlorocruorin relative to oxy- and reduced haemoglobin.

9. The porphyrin prepared from chlorocruorin is either identical with haemato-porphyrin or very closely resembles the latter.

10. Chlorocruoroporphyrin, like haematoporphyrin, has a brilliant red fluorescence.

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THE EFFECT OF LIGHT ON THE VERTICAL MOVEMENT OF AQUATIC ORGANISMS

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(Received 11 September 1924.)

(With Five Text-figures.)

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1. INTRODUCTION.

THE investigation described below was commenced in March 1920 at the School of Medicine, Cairo, with the observation that *Paramecium* in a vertical glass tube under certain conditions swims downwards in light and upwards in darkness. It seemed probable that were this a general reaction of aquatic organisms it might be a more important causative factor in the diurnal vertical migrations of plankton than the change of phototropism with changing *pH* and temperature which Loeb (1894) put forward as the principal cause. It is known that many animals in the plankton are found nearer the surface during the hours of darkness, lower down in the daytime. Loeb (1893 and 1906) showed that plankton crustacea are made positively phototropic by a low temperature and by a low *pH*. Now the water temperature and the *pH* in the sea may be higher in the daytime than at night, the *pH* owing to photosynthesis by plankton algae (Legendre, 1922). By a consequent reversal of phototropism from day to night Loeb explained the diurnal vertical migrations. But apart from the probability that under the conditions of illumination found in the sea phototropism is ineffective (Rose, 1924, p. 6), for the intensity of orientated light is not sufficiently superior to that of diffuse light, it is difficult to understand why the plankton should move up at night when there is no light to cause any phototropism at all. It appeared to me therefore that the downward movement of *Paramecium* in light and the upward movement in darkness if applicable to plankton organisms would be a more important cause of the daily up and down migration.

During the summer of 1920 at Suez I confirmed the light effect on vertical distribution for certain plankton organisms notably echinoid larvae and crustacea. After concluding the experimental work however I found that owing to my isolation from libraries I had missed work already published upon the same subject; in

particular Esterley (1907 and 1919) had found the light effect on the geotropism of copepods, Harper (1907, p. 447) of *Corethra* larvae, Menke (1911) of crustacea, and Dice (1914) of *Daphnia*. Further, these authors point out how the reaction must be an important causative factor in the diurnal vertical movements of plankton.

It may nevertheless be of interest to record my own observations as they show that the phenomenon is a very general one, existing both in animals swimming by muscular movements such as arthropods and in those moving by ciliary action as echinoid larvae and ciliates. In addition, observations made in 1924 at the Roscoff Biological Station are recorded indicating what part of the spectrum is concerned.

2. EXPERIMENTS WITH *PARAMECIUM*.

Paramecium in a vertical tube* moves either upwards or downwards according to a number of circumstances. Before studying the effect of light and darkness on vertical movement it was necessary to investigate other factors concerned which may partially mask the light effect. The most important are the two following.

(1) Mechanical shock produced by rapid pouring of the suspension of organisms into the experimental tubes causes an immediate downward movement with a consequent accumulation at the bottom, which however is transitory.

(2) In their own culture fluid *Paramecium* swims to the top of the experimental tubes whereas in tap water it goes to the bottom. The culture fluid was tap water containing abundant living and dead water snails. In order to transfer *Paramecium* to pure tap water the organisms were pipetted from a top or bottom aggregation in their culture fluid and then placed in the tap water.

The culture fluid differed from the Cairo tap water used by a number of factors which might have been responsible for the behaviour of the *Paramecium*. The question was not followed up further, since for the study of the light effect on vertical movement it was sufficient to take into account the difference between the behaviour in tap water and in culture fluid. It is possible however that a higher CO_2 content of the culture fluid may be the responsible factor. Koehler (1922, p. 34) has shown that increased CO_2 tension causes an upward movement of *Paramecium* while changed O_2 tension has no effect. I can confirm Koehler's observation. Further I have found that the aeration of a suspension of *Paramecium* in culture fluid, brought about by leaving the liquid exposed in petri dishes for some time before filling the experimental tubes, causes an initial downward movement with aggregation at the bottom of the tube, before the usual upward movement in the culture fluid sets in. This behaviour too may well be due to a diminution in CO_2 content brought about by exposure to air in the petri dish.

Coming now to the main experiments on the effect of light and darkness, it was found that illumination causes downward movement, darkness upward movement. *Paramecium* which, in a dull light, is evenly distributed throughout the water of a vertical glass tube moves upwards when placed in darkness and downwards in a bright light. In culture fluid, which imposes upward movement, the

* The experimental glass tubes were completely filled with liquid and corked above to avoid a water-air interface and consequent O_2 or CO_2 gradient.

aggregation at the upper end of the vertical tube is more crowded in darkness, less so in light. In tap water, which causes downward movement, the bottom aggregation is more dense in light than in darkness. These points are illustrated by

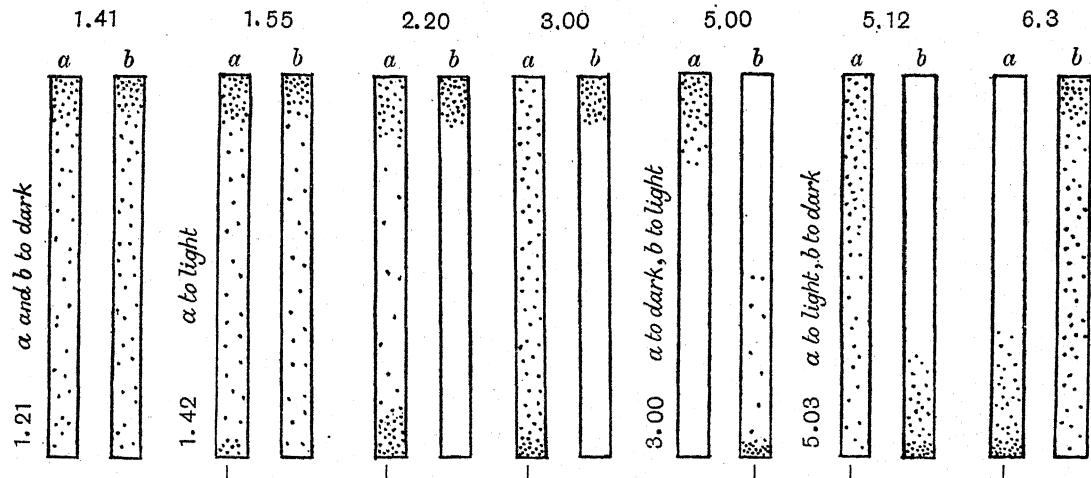


Fig. 1. (26. 3. 21.) *Paramecium* in culture fluid diluted with tap water. Two tubes (a and b) placed in darkness at 1.21 p.m. The tubes measured 24×1 cm. and were corked at both ends to avoid a water-air interface. In all diagrams the density of dots represents the density of distribution of the organisms. Tubes marked L were in bright diffuse daylight, tubes not so marked were in darkness.

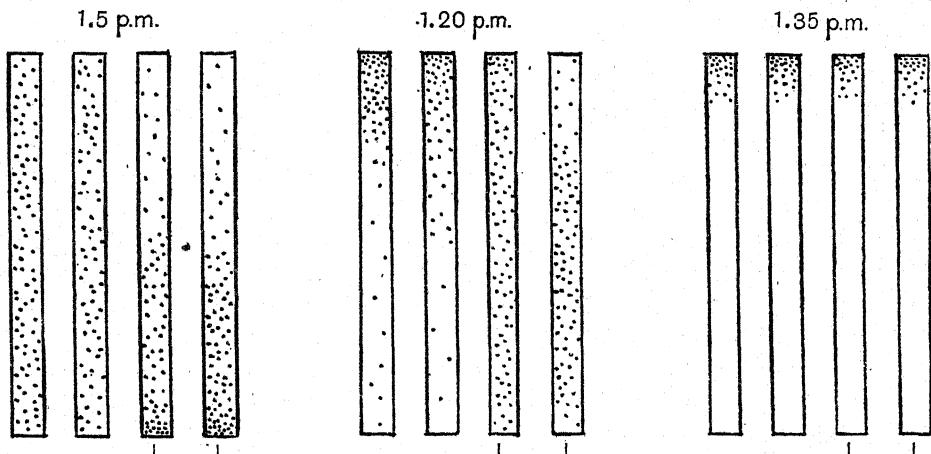


Fig. 2. (22. 3. 21.) *Paramecium* in undiluted culture fluid. Two tubes put in bright diffuse daylight and two others in darkness at 12.40 p.m. Tubes marked L were in light, tubes not so marked were in darkness. Tube dimensions as in Fig. 1.

Figs. 1 and 2 which represent typical experiments out of an extensive series. The light effect was transitory in Fig. 2 because the experiment was done in undiluted culture fluid which induces upward movement.

This effect of light on up and down movement is independent of any phototropism. To be certain of this, additional experiments were made with the setting

sun so that the rays were perpendicular to the glass tubes. The result was the same as before. Moreover *Paramecium* is not phototropic to visible light at all although it is negatively phototropic to the ultra-violet (Hertel, 1904, and Metzner, 1921, p. 150). It might be thought that the sinking of the *Paramecium* in the light was passive and due to a slowing of the ciliary movement. This is not the case however. Observed on slides on the microscope stage the rate of swimming is slightly faster in direct sunlight than in dull light. This confirms Metzner (1921, p. 149). Nor is the light effect on vertical migration due to a rise in temperature due to the radiation. To test this possibility four tubes were placed in darkness, two at 23° and two at 31°. The final result was identical at both temperatures, an aggregation at the top of the tubes. At 31° however there was an initial downward swimming absent at 23°. After this the *Paramecium* at 31° rose more rapidly than at 23°.

3. EXPERIMENTS WITH ECHINOID LARVAE.

The larvae of *Diadema (Centrechinus) setosum* were used. They were obtained at Suez by artificial insemination. The effect of illumination on vertical movement was found to be the same as for *Paramecium*. Fig. 3 shows that the blastulae display the phenomenon. Gastrulae and plutei behave in the same way.

As in the case of *Paramecium* it was necessary to be certain that negative phototropism was not a partial cause of the sinking in light. For this purpose tubes were placed in a wide black paper cylinder closed above and sunlight was reflected by a mirror from below into the cylinder. This illumination caused the usual downward movement of the plutei in the tubes although the movement in this case was towards the light.

Preliminary experiments had shown that the *pH* of the water has a strong influence on the upward or downward movement of the larvae. The addition of acid to the sea-water causes upward, of alkali downward movement. Carbonic is a sufficiently strong acid to give the upward migration. In two tubes containing plutei air was bubbled through one and CO₂ through the other. When the bubbling had ceased the larvae remained evenly scattered throughout the air tube whereas they rose to the surface of the CO₂ tube. This is identical with the effect of CO₂ on the vertical movement of *Paramecium* described above.

Fig. 4 illustrates an experiment with plutei in which both the *pH* and the light effects are exhibited. The acid water was prepared by adding 0.02 c.c. of 1*N* HCl to 10 c.c. sea-water, the alkaline by adding 0.02 c.c. of 1*N* NaOH to 10 c.c. sea-water. It is evident that the light takes much longer to act than the *pH* the effect of which is very rapid. At 11.37 the light effect was not yet apparent. When the

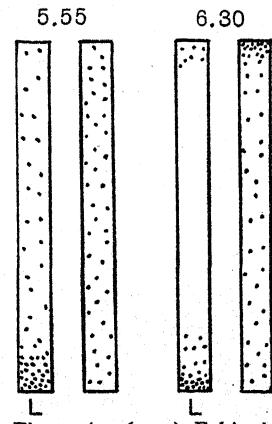


Fig. 3. (22.6.20.) Echinoid blastulae. One tube placed in light another in darkness at 5.35 p.m. Tubes marked L were in sunlight, tubes not so marked were in darkness. Tube dimensions 53 x 1 cm.

light and the *pH* act in opposite directions the light effect is partially masked, as in the acid tubes in light at 3.50.

The mechanism of the downward movement requires further investigation. Whether moving upwards or downwards plutei swim always with the aboral pole down and the arms upwards. They rotate rapidly and continuously on the vertical axis when young, less rapidly and discontinuously when older.

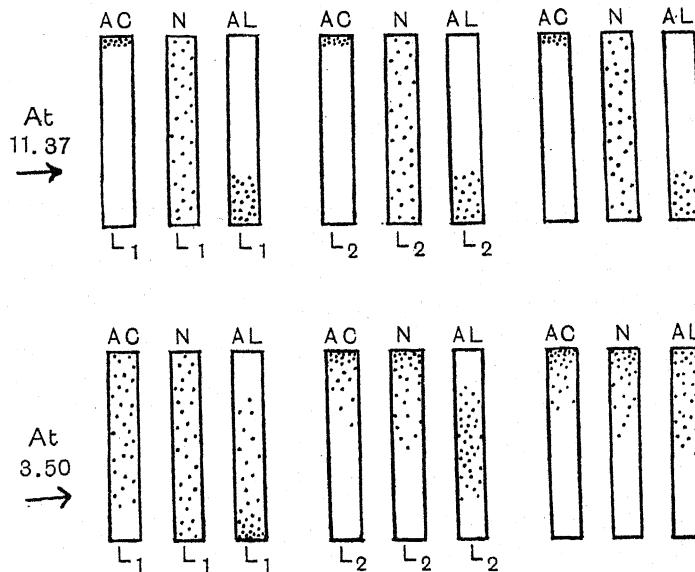


Fig. 4. (22.7.20.) Plutei in test tubes with oil on surface of water. Experiment commenced 11.24 a.m. AC: acid; N: normal; AL: alkaline water. Tubes marked L₁, in bright diffuse daylight, those marked L₂ in dull diffuse daylight, the remainder in darkness.

4. THE EFFECTIVE REGION OF THE SPECTRUM.

Since *Paramecium* are unpigmented and echinoid larvae almost so it seemed very probable that the influence of light on vertical movement is due to the ultra-violet. That this is actually the case was shown at Roscoff this summer with larvae of the sea-urchin *Paracentrotus lividus*. Comparative experiments were made out of doors in corked test-tubes made of silica and of glass. The light effect was much more marked in the silica tubes. This is shown by Fig. 5.

Ultra-violet light of sufficient intensity and duration destroys protoplasm. It was possible therefore that the sinking of plutei in the lighted silica tubes was an incipient death and was irreversible. That such is not the case is shown by the second part of the experiment illustrated in Fig. 5. The plutei rose again from the bottom in the tubes which had been in the light and were put into darkness at 12.5 p.m.

It is known that colourless organisms such as *Paramecium* which are normally uninjured by light are killed by light in the presence of fluorescent substance (Tappeneier). Further *Paramecium* which normally shows no phototropism behind glass

becomes phototropic in the presence of fluorescent substances (Metzner, 1921). The influence of such substances on the light effect on vertical movement was tried at Roscoff with plutei and it was found that the fluorescent substances augment the light effect. Eosin 1 : 20,000 and erythrosin A and B 1 : 50,000 were used. The sinking in light was again reversible, for when the tubes were removed from light to dark the plutei rose.

5. SUMMARY.

1. *Paramecium* and echinoid larvae under certain environmental conditions swim downwards in light, upwards in darkness. Thus the phenomenon already described by various authors for arthropods exists not only in animals swimming by muscular movements but in organisms moving by ciliary action.

2. The phenomenon is reversible.

3. The most effective rays are in the ultra-violet.

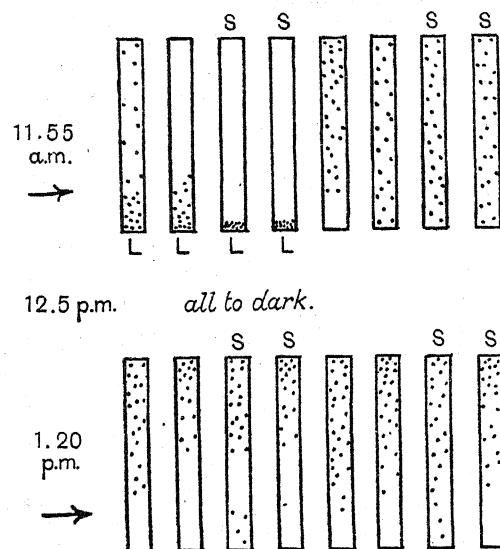


Fig. 5. (12.7.24). Plutei in corked test tubes. Experiment commenced 11.23 a.m. Tubes marked S were of silica, tubes not so marked were of glass. Tubes marked L in diffuse daylight, the remainder in darkness.

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THE MECHANISM OF CELL-DIVISION

II. OXYGEN CONSUMPTION DURING CLEAVAGE.

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(Received 10 August, 1924.)

(With Three Text-figures.)

In a previous paper (Gray⁽³⁾) it has been shown that the cleavage of echinoderm eggs depends upon the position and on the size of the asters which accompany the mitotic division of the nucleus. The cell cleaves at the moment the asters have reached their maximum size, and when the extremities of the astral rays lie very near the periphery of the cell. For this and other reasons, it follows that cleavage is the necessary conclusion to the process of aster formation. This goes on continuously during the whole mitotic cycle, and does not involve a sudden change in the surface energy of the cell at its equator, nor any contractile function on the part of the astral rays. Cleavage is essentially due to a rearrangement of the different phases round the two growing asters. It is obvious that this conclusion is at variance with the views put forward by McClendon⁽¹¹⁾, Robertson⁽¹⁴⁾, Spek⁽¹⁸⁾ and Heidenhain⁽⁴⁾. Although the schemes suggested by the first three authors differ materially from that of Heidenhain, yet all postulate some sudden activity on the part of the cell, either by the liberation of a substance at the equator of the cell or by actual contraction of the astral rays. In support of these suggestions there is no direct experimental evidence. If the fully developed rays are capable of suddenly developing a tension sufficient to cleave the cell, the nearest comparison to them would be the contractile fibres of a muscle. If, on the other hand, cleavage be due to the liberation of some substance capable of producing a very marked change in the surface energy on part of the cell-surface, then some mechanism must exist for the formation of this substance, and for its ultimate removal from the cell. In either case, but in the first case in particular, one might expect that the sudden activity of the cleaving mechanism would be marked by a change in the observable metabolism of the cell. Although, hitherto, no data have been available concerning the oxygen consumption during cleavage, an attempt was made in 1904 by Lyon⁽⁹⁾ to measure the carbon-dioxide output of the cleaving eggs of *Arbacia*, and more recently (1922) Vlès⁽²⁰⁾ has measured by an indirect method that from the eggs of *Paracentrotus lividus*.

Lyon⁽⁹⁾ found that

"in nearly all experiments there was an increase in CO_2 production during the first ten or fifteen minute interval following fertilisation. The increase was slight and sometimes could not be detected. Following this came an interval in which the CO_2 production

was small, visibly less indeed in two or three experiments than that of the unfertilised eggs and sperm. This is the mid-period of cleavage, approximating perhaps the time of nuclear growth and the early stages of karyokinesis. The interval during which the eggs were dividing into the first two blastomeres was one of active CO_2 production. After this period came an interval of lessened production. In one or two cases a second rise occurred at about the time of the second cleavage."

In a later paper⁽¹⁰⁾ dealing with the susceptibility of the egg to various reagents, he refers to these experiments as follows:

"It may be stated that the apparent conclusion was that CO_2 production in the egg is not uniform throughout the whole series of morphological changes of cell division, but rather reaches a maximum at the time when the cytoplasm is actively dividing. Furthermore it seemed that at the time when O_2 is most necessary and presumably is being used in largest amount (as indicated by susceptibility to lack of O_2 and to KCN) CO_2 is produced in least amount. If the conclusion above expressed should justify itself it would indicate that oxygen is chiefly used in the egg for synthesis rather than for combustion, and that the larger part of the CO_2 comes from splitting processes. One would also infer that the energy for cell-division comes from fermentative rather than oxidative processes."

Lyon makes it abundantly clear, however, that he does not regard his experiments as of sufficient accuracy to permit of reliable theoretical treatment.

"These statements may need revision in the light of later and more accurate investigations."

As far as I know, these have not been forthcoming.

By observing the change in the p_{H} of the fluid in contact with the eggs of *Paracentrotus* Vlès⁽²⁰⁾ found that well marked cyclical changes occur, and he attributes this, at least in part, to a periodic liberation of CO_2 . Unless these observations are really due to CO_2 output, they are of course useless as a basis for determining energy changes in the cell. In the present paper it will be assumed that Vlès' observations are not vitiated by the evolution or absorption of any other ions apart from those of CO_2 , since positive evidence to the contrary is not apparent. Such difficulties are, of course, avoided by estimating the oxygen consumption of the cells.

It therefore seemed to be desirable to investigate the respiration of cleaving cells by the direct measurement of the oxygen consumed.

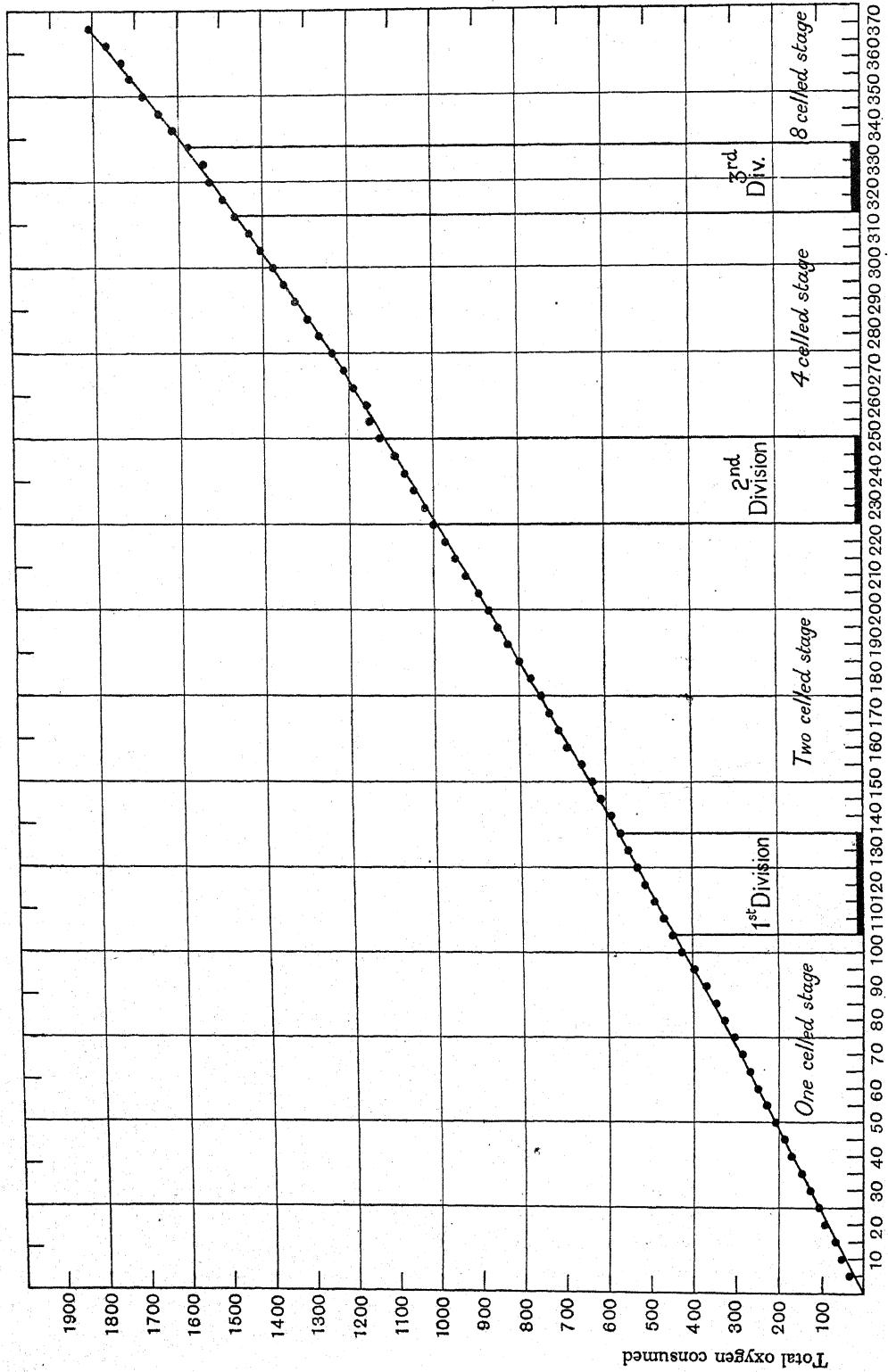
The material used for the following experiments was the eggs of *Echinus esculentus*. In order that an accurate estimation of the rate of oxygen consumption may be made at definite stages during the whole mitotic cycle, it is necessary that the conditions of the experiment should be such as will allow the eggs to develop normally and at the same rate, so that practically all the cells cleave at the same time. Again, in order that several determinations can be made during the comparatively short time occupied by the cleavage process, it is advisable to prolong this period by carrying out the whole experiment at a fairly low temperature. It was found that at 11°C . the time required for the first cleavage was about half-an-hour; that is to say, it takes half-an-hour from the time the eggs first begin to show traces of a cleavage furrow, until all the cells have completely divided into two blastomeres. The time required for subsequent cleavages is shorter.

A typical experiment was performed as follows:—The eggs from a ripe female were fertilised, and washed repeatedly in clean sea-water. They were then divided into two approximately equal portions, and each portion was concentrated into 6 c.c. of sea-water and placed in the respiration chamber of a Barcroft respirometer. The manometers were kept continuously shaken and the bottles kept in a bath of water at 11° C. The form of respiration bottle used was of the Erlenmeyer type and had a volume of 28 c.c. After about 20 minutes the taps were turned so as to place the bottles in communication with the manometers. In the case of one apparatus, the excursion of the meniscus was read every five minutes; the other apparatus was kept as a control whereby to follow the process of development. By removing eggs from this control apparatus, the stage reached by the eggs can be followed, and from time to time the stage of development can be compared with that reached in the apparatus used for determining the oxygen consumption. At the beginning of this work it was thought that with a large number of eggs developing in a small bulk of water, the development would tend to be very irregular and slow. This was found not to be the case, and is no doubt due to the fact that the eggs were continuously shaken, and that the CO₂ was being completely absorbed by the caustic potash in the apparatus. Further, at fairly low temperatures it is easy to supply sufficient oxygen by gentle agitation. In all the experiments it was found that the eggs in both pieces of apparatus used for an experiment developed at the same rate, and that this was only slightly slower than isolated eggs immersed in a large bulk of sea-water.

It is of importance to know with what degree of accuracy to regard the observed figures. Two sources of error occur. One is due to the error in reading the meniscus; this has been found by practise to be of the order of ± 1 mm. The other source of error lies in irregularities of the manometer when working under low pressures. It is difficult, even with thoroughly clean manometer tubes, to prevent occasional "lags" in the meniscus, but if a low reading is rapidly followed by one above the average, one may be tolerably certain that the cause lies in the meniscus. This error is spasmodic, and has an approximate value of ± 2 mm. Individual readings can, therefore, only be regarded as correct to within these errors. It has been found that the maximum probable error when measuring the respiration of tissues with a steady rate of respiration seldom exceeds ± 3.5 mm.

Table I gives the data derived from one experiment, and Fig. 1 shows graphically the total oxygen consumption in the same experiment plotted against time. Fig. 2 shows the actually observed amount of oxygen consumed per 5 mins. during the period occupied by three successive cleavages. Fig. 3 shows similar data from another experiment.

An examination of Fig. 1 shows that the amount of oxygen consumed is not a linear function of the time. After the second division has occurred there is distinct evidence of an acceleration in oxygen consumption although this acceleration is independent of the process of cell-division. By taking the slope of the curve in Fig. 1 at successive periods and plotting the points so derived on to Fig. 2 the thick



Time in minutes from the beginning of the experiment

Fig. 1. Graph showing the total oxygen absorbed by the fertilised eggs of *Echinus esculentus* during three successive divisions. The black lines on the time axis indicate the periods occupied by the process of cleavage. Experiment A.

line *AB* (Fig. 2) is obtained as the theoretical change in rate during the whole period of the experiment. This curve conforms to the conditions that the logarithm of the rate of respiration is proportional to the time. From this it is possible to

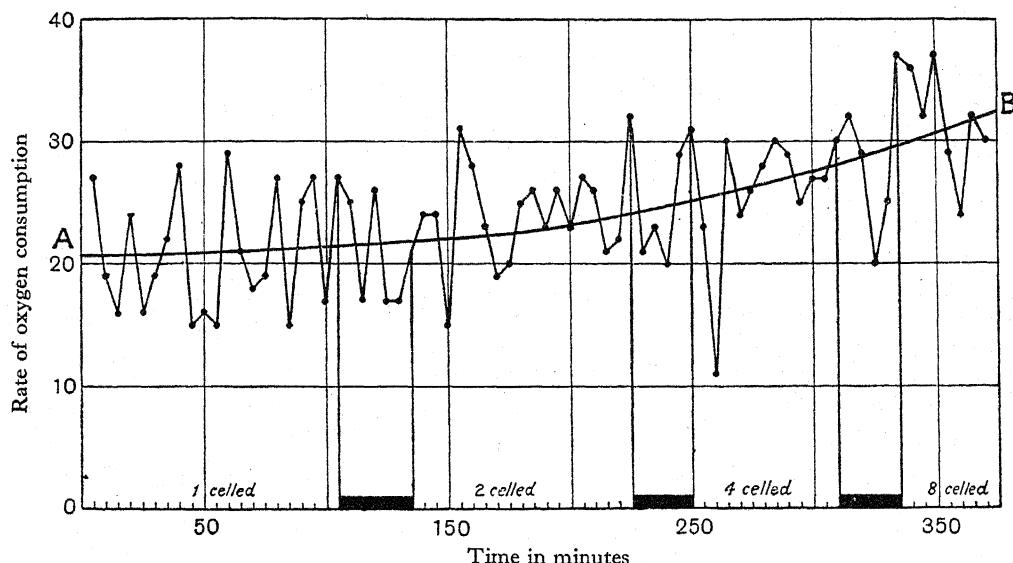


Fig. 2. Graph showing the rate of oxygen absorption during the process of cleavage. *AB* shows the rate equivalent to the smooth curve in Fig. 1. Experiment A.

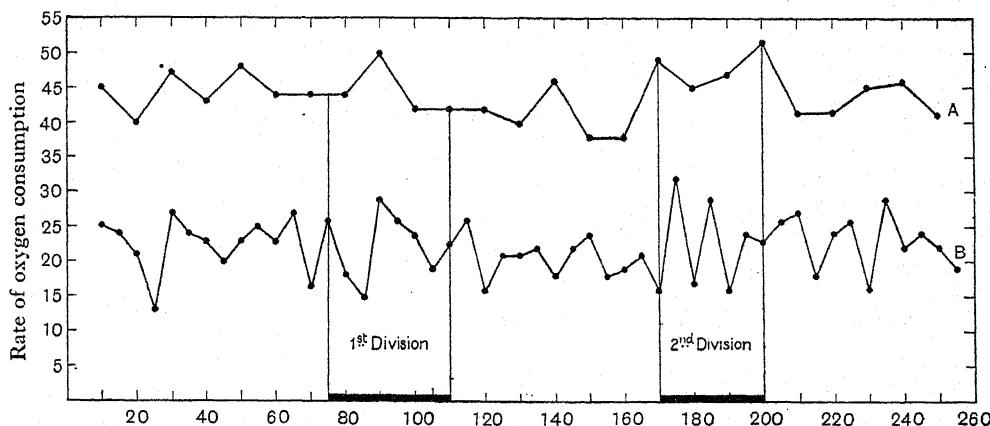


Fig. 3. Graph showing the rate of oxygen absorption during the process of cleavage. Experiment B.

calculate the theoretical value of the total oxygen consumption at any moment. These figures are shown in Table I and agree very closely with the observed readings.

A consideration of Figs. 2 and 3 shows that the fluctuations in the rate of oxygen consumption bear no relationship to the periods of cleavage. If an allowance

Table I
Details of Experiment A.

Eggs fertilised 10.45 a.m. After thorough washing, eggs transferred to apparatus at 12.45 a.m.
 (zygote nucleus stage). $T = 11.0^{\circ}$.

Time in minutes from beginning of exp.	Total O_2 consumed			Amount of O_2 consumed in 5 minutes			State of cleavage
	Obs.	Calc.	Error	Obs.	Calc.	Error	
5	2.7	2.1	+0.6	2.7	2.1	+0.6	
10	4.6	4.2	+0.4	1.9	2.1	-0.2	
15	6.2	6.3	-0.1	1.6	2.1	-0.5	
20	8.6	8.4	+0.2	2.4	2.1	+0.3	
25	10.2	10.5	-0.3	1.6	2.1	-0.5	
30	12.1	12.6	-0.5	1.9	2.1	-0.2	
35	14.3	14.7	-0.4	2.2	2.1	+0.1	
40	17.1	16.8	+0.3	2.8	2.1	+0.7	
45	18.6	18.9	-0.6	1.5	2.1	-0.6	
50	20.2	21.1	-0.9	1.6	2.1	-0.5	
55	21.7	23.2	-1.5	1.5	2.1	-0.6	
60	24.6	25.4	-0.8	2.9	2.1	+0.8	
65	26.7	27.5	-0.8	2.1	2.1	0	
70	28.5	29.6	-1.1	1.8	2.1	-0.3	
75	30.4	31.7	-1.3	1.9	2.1	-0.2	
80	33.1	33.8	-0.9	2.7	2.1	+0.6	
85	34.6	36.0	-1.4	1.5	2.2	-0.7	
90	37.1	38.2	-1.1	2.5	2.2	+0.3	
95	39.8	40.4	-0.6	2.7	2.2	+0.5	
100	41.5	42.6	-1.1	1.7	2.2	-0.5	
105	44.2	44.8	-0.6	2.7	2.2	+0.5	
110	46.7	47.0	-0.3	2.5	2.2	+0.3	
115	48.4	49.2	-0.8	1.7	2.2	-0.5	
120	51.0	51.4	-0.4	2.6	2.2	+0.4	
125	52.7	53.6	-0.9	1.7	2.2	-0.5	
130	54.4	55.8	-1.4	1.7	2.2	-0.5	
135	56.5	58.0	-1.5	2.1	2.2	-0.1	
140	58.9	60.3	-0.4	2.4	2.3	+0.1	
145	61.2	62.6	-1.4	2.4	2.3	+0.1	
150	62.8	64.9	-2.1	1.6	2.3	-0.7	
155	65.9	67.2	-1.3	3.1	2.3	+0.8	
160	68.7	69.5	-0.8	2.8	2.3	+0.5	
165	71.0	71.8	-1.7	2.3	2.3	0	
170	72.9	74.1	-1.2	1.9	2.3	-0.4	
175	74.9	76.4	-1.5	2.0	2.3	-0.3	
180	77.4	78.7	-1.3	2.5	2.3	+0.2	
185	80.0	81.0	-1.0	2.6	2.3	+0.3	
190	82.3	83.4	-1.1	2.3	2.4	-0.1	
195	84.9	85.8	-0.9	2.6	2.4	+0.2	
200	87.2	88.2	-1.0	2.3	2.4	-0.1	
205	89.9	90.6	-0.7	2.7	2.4	+0.3	
210	92.5	93.0	-0.5	2.6	2.4	+0.2	
215	94.6	95.4	-0.8	2.1	2.4	-0.3	
220	96.8	97.8	-1.0	2.2	2.4	-0.2	
225	100.0	100.2	-0.2	3.2	2.4	+0.8	
230	102.1	102.7	-0.6	2.1	2.5	-0.4	
235	104.4	105.2	-0.8	2.3	2.5	-0.2	
240	106.4	107.7	-1.3	2.0	2.5	-0.5	
245	109.3	110.2	-0.9	2.9	2.5	+0.4	
250	112.4	112.7	-0.3	2.6	2.5	+0.1	
255	114.7	115.3	-0.6	3.0	2.6	+0.4	
260	115.8	117.9	-2.1	1.1	2.6	-1.5	
265	118.8	120.5	-1.7	3.0	2.6	+0.4	
270	121.2	123.1	-1.9	2.4	2.6	-0.2	

Table I (contd.)

Time in minutes from beginning of exp.	Total O ₂ consumed			Amount of O ₂ consumed in 5 minutes			State of cleavage
	Obs.	Calc.	Error	Obs.	Calc.	Error	
275	123.8	125.7	-1.9	2.6	2.6	0	
280	126.6	128.4	-1.8	2.8	2.7	+0.1	
285	129.6	131.1	-1.5	3.0	2.7	+0.3	
290	132.5	133.8	-1.3	2.9	2.7	+0.2	
295	135.0	136.5	-1.5	2.5	2.7	-0.2	
300	137.7	139.2	-1.5	2.7	2.7	0	
305	140.4	142.0	-1.6	2.7	2.8	-0.1	
310	143.4	144.8	-1.4	3.0	2.8	+0.2	
315	146.6	147.6	-1.0	3.2	2.8	+0.4	
320	148.6	150.5	-1.9	2.0	2.9	-0.9	
325	151.5	153.4	-1.9	2.9	2.9	0	
330	154.0	156.3	-2.3	2.5	2.9	-0.4	
335	157.7	159.3	-2.0	3.7	3.0	+0.7	
340	161.3	162.3	-1.0	3.6	3.0	+0.6	
345	164.5	165.3	-0.8	3.2	3.0	+0.2	
350	168.2	168.4	-0.2	3.7	3.1	+0.6	
355	171.1	171.5	-0.4	2.9	3.1	-0.2	
360	173.5	174.7	-1.2	2.4	3.2	-0.8	
365	176.7	177.9	-1.2	3.2	3.2	0	
370	180.7	181.1	-0.4	4.0	3.2	+0.8	

The oxygen is shown as millimetres pressure of O₂ and can be converted into cubic millimetres of volume by multiplying by 2.5.

be made for the probable error of each determination, the large majority of the observed fluctuations are eliminated, whilst those which remain are irregular in their occurrence and are of very short duration. It may therefore be inferred that if the process of cleavage involves any change in the oxygen consumption of the cell, this change is so small and of such short duration that the total amount of oxygen involved can only be a very small fraction of the total oxygen being used by the cell.

Again, the following figures, Table II, show that the oxygen consumption during the half hour periods prior to, during, and after the first two divisions is very nearly the same.

Table II

Mm pressure O₂ used per half hour

No. of exp.	Immediately previous to division	During division	Immediately after division	No. of division
A	13.0	12.3	14.0	1st
A	14.8	15.0	13.7	2nd
A	16.4	15.9	18.3	3rd
B	14.1	13.6	13.0	1st
B	13.3	13.4	13.7	2nd
C	6.5	6.1	7.1	1st
D	21.0	19.0	21.0	1st
Totals 99.1		95.3	100.8	

In view of the above results it is necessary to investigate the fact, established by Lyon (8) and by Matthews (12) that echinoderm eggs exhibit a marked periodicity in their susceptibility to cyanide poisoning. From the evidence given by these authors there can be little or no doubt that prior to fertilisation the egg is relatively resistant to cyanide. For the first 20 minutes after fertilisation, on the other hand, the eggs of *Arbacia* are relatively sensitive; after this period the resistance rises and no further susceptibility is found until immediately before and during each of the cell-cleavages. Loeb (7) found that in the absence of oxygen cleavage did not occur, and Matthews found that reagents such as cold, quinine, and anaesthetics which are known to reduce oxidations, also prevent cell-division and cause a disappearance of the astral rays. On this evidence Matthews (12) concluded that the whole process of cell-division is intimately associated with the oxidative processes in the egg, and that the periodicity of the former is due to a periodicity in the capacity of the cell to carry out certain oxidations involving the use of atmospheric oxygen, this periodicity in oxidative power being due to the periodic liberation from the nucleus of an oxidase whenever the nuclear membrane breaks down. In opposition to this conclusion is the fact established by Warburg (21) that the level of oxygen consumption of the eggs can be maintained almost unchanged when the periodic changes of the nucleus are entirely inhibited.

The paradox presented by these facts is, in my opinion, only apparent. In the first place the periodic susceptibility of echinoderm eggs to cyanide is exactly paralleled by that of the eggs to a variety of reagents having little or no obvious direct effect on the respiration. A similar periodicity to that shown by Lyon for cyanides has been shown to exist by Spaulding (17) for ether, heat and hypertonic sea-water; by R. S. Lillie (6) for hypotonic sea-water; and by Baldwin (1) for alcohols. Now it has been shown in a previous paper (Gray (3)) that for the first 15-20 mins. after fertilisation the surface of the fertilised egg is undergoing an active change in the differentiation of a definitive ectoplasm or cortical layer. This layer is fully formed after about 30 mins., and remains equally distributed over the egg surface until just before the appearance of the cleavage furrow. During the process of cleavage there is a marked change in the distribution of this surface layer. It accumulates at the equator and becomes very thin at the poles. After cleavage the polar ectoplasm thickens again but never reaches its original thickness. The two periods of susceptibility to reagents share, therefore, the characteristic that the surface layer is abnormally thin either over all the egg surface or over its poles. Just (5) and Lillie (6) have both observed that the region of the cleaving egg most susceptible to reagents lies at the surface of each pole. There is, therefore, some ground for believing that the properties of the surface layer are such as to inhibit the effect of reagents in general, including KCN^* . This is confirmed by the fact that the final effect of KCN on the blastomeres of *Ctenolabrus* is to cause

* A peculiarly interesting example of the instability of the polar surfaces of dividing cells is described by Strangeways (19). In certain cells the surface layer at the poles appears to rupture temporarily as soon as the cell begins to cleave. According to Strangeways these cells have no visible asters. It would be interesting to know whether microdissection would reveal two enlarging areas of high viscosity comparable to the asters of echinoderm eggs, since the astral rays of the latter are probably not the direct cause of the higher viscosity of the region they occupy.

them to fuse together—and since the cortical layer prevents this in the normal egg (see Gray⁽³⁾), it is fairly clear that the initial effect of KCN is to render soluble this surface layer. Until the surface layer is fully formed and whilst it is temporarily reduced at the poles of the cell the egg of the sea-urchin is more susceptible to reagents than at intermediate times. This is again confirmed by the fact that with successive divisions the thickness of the ectoplasm is reduced, and at the same time the sensitivity to KCN increases (Lyon⁽⁸⁾).

Certain authors, including Matthews⁽¹²⁾, have developed attractive hypotheses of the mechanics of cell-division from the evidence of the effect of KCN. Whilst it is true that this reagent depresses the oxidations in echinoderm eggs, it does not abolish it. Loeb and Wasteney's^(7a) figures show that a 0.0003% solution of KCN reduced the respiration to about one-third of its normal value; but the distinctive property of KCN is that although very dilute solutions have a detectable effect on the oxidations, successive increases in the concentration of KCN have less and less effect, and it is impossible to reduce the respiration of tissues below about 20% of the normal. This maximum effect is reached by $50 \times 10^{-6} M$. KCN, Gray⁽²⁾. If we compare this concentration with those used by Lyon to demonstrate the susceptibility of sea-urchin eggs, viz. $\frac{M}{50} - \frac{M}{200}$, it becomes obvious that the KCN used in this latter connection is operating in some way other than its direct effect on the oxidation. This fact is of interest when attempts are made to correlate susceptibility to KCN and oxygen consumption.

Lyon showed that for the first 15 mins. after fertilisation the eggs of *Arbacia* are abnormally sensitive to a lack of atmospheric oxygen, and, as mentioned above, at a later stage an absence of oxygen causes individual blastomeres to fuse together. It therefore looks as though the surface layer of the cell is only stable in the presence of oxygen. It is hoped that further work will throw light on this interesting phenomenon.

The nature of the rhythm exhibited by all these reagents is, however, of a different nature to that described by Vlès for the evolution of CO_2 . In this case the period of maximum CO_2 evolution comes apparently immediately after each cleavage, and the rate falls off gradually during the next mitotic cycle. The graph given by Vlès does not, unfortunately, give sufficiently accurate data to determine the actual rate of CO_2 production at different periods of time. The figure published by him is, however, comparable to Fig. 1 of this paper. It is at once obvious that the marked periodicity observed by Vlès is not accompanied by a corresponding periodicity of oxygen consumption. The variations in the rate of CO_2 production at different periods are of an altogether different order to the very slight and transient variations in oxygen consumption.

It may perhaps be permissible to point out one or two peculiarities in Vlès' curve. It would appear that just prior to the third cleavage there is an actual absorption of CO_2 , unless this point is due to experimental error. Again, after the third cleavage, the eggs do not appear to have given off any CO_2 for more than one hour. Before attaching implicit faith to these facts one would like to know more precisely the conditions under which the experiment was carried out. In experiments

dealing with small variations in the respiration of cells it is essential that the conditions should be accurately defined. For this reason, the results of Vlès cannot strictly be compared to those here described. In Vlès' experiments the CO_2 was allowed to accumulate, and the eggs were not agitated*. In the present work the CO_2 was continuously removed and the solution saturated with oxygen by agitation. On general grounds there can be little doubt that the oxygen consumption is a more reliable guide to the metabolic activity of the eggs than is the evolution of CO_2 , particularly when the latter is determined by an indirect method. At the same time there is no *a priori* reason why the CO_2 output should bear a definite relationship to the O_2 consumption except over prolonged periods. If one considers the newly fertilised egg, it is reasonably clear from the work of Shearer⁽¹⁶⁾ that the rate of O_2 consumption fairly rapidly attains its new level. The amount of CO_2 which will escape during this period will possibly depend upon three factors: (1) the alkali reserve of the egg, (2) the alkalinity of the surrounding medium, and (3) the surface area of the egg. Eventually one would expect an equilibrium in CO_2 output to be reached, so that the latter will bear a definite relation to the O_2 consumption. When cleavage occurs this equilibrium may be disturbed by the fact that the surface of the egg has increased, and some of the CO_2 held in the egg may be free to escape. That there is a lag between the O_2 consumption and the CO_2 output has been shown to accompany sudden increases in the activity of other tissues (Gray⁽²⁾). Whether such causes play any part in the case of echinoderm eggs is impossible to say from the data at present available.

It is clear, however, that since the periodicity in evolution of CO_2 is not accompanied by a periodicity in O_2 uptake, the former cannot be regarded as of significance in respect to energy changes in the egg, unless they be due to some obscure form of anaerobic activity for which there is no evidence. It will have been noticed perhaps that the periodicity in CO_2 production reported by Vlès does not correspond to that described by Lyon. In the former case the period of maximum CO_2 evolution is immediately after the division, in the latter case this period is one of reduced CO_2 evolution.

It is just possible that the process of aster formation involves the use of CO_2 , or that some associated process prevents the loss of CO_2 from the egg. Such conditions would, of course, lead to a periodic liberation of CO_2 whenever the asters faded away, and would be in harmony with some of the data given by Vlès.

It has been claimed that the nucleus plays an active rôle in the oxidative mechanism of the cell (Matthews⁽¹²⁾, Osterhout⁽¹³⁾). The present experiments show that if this is the case, the presence of a nuclear membrane or definitive nucleus is unnecessary since the rate of oxidation is independent of the phase of nuclear activity.

In a subsequent paper, the steady increase in the rate of oxygen consumption during the first few hours of development will be considered in more detail. It is, however, already clear that this increase is independent of the phases through which the nuclei are passing. This fact has a direct bearing on an ingenious hypothesis put

* Personal communication.

forward by Robertson (15). This author suggests that the possibility for cell-division and growth is dependent upon the relative distribution of a catalyst between the nucleus and the cytoplasm of the cell. This autocatalyst is formed within the nucleus and can only escape into the cytoplasm when the nuclear membrane breaks down at each prophase. Although Robertson does not suggest that this autocatalyst is actually an oxidising agent, yet he does accept the view that the distribution of this substance can satisfactorily be followed by the rate of cellular respiration. The autocatalyst increases within the resting nucleus and is only shared with the cytoplasm when the nuclear membrane breaks down. If this be so, one would undoubtedly expect to find a periodic change in the oxygen consumption. This is not the case.

One other point remains to be considered. It is clear that the independence of cleavage and the rate of oxygen consumption indicates that the asters are not using up oxygen at a rate proportional to their volume, and that their rôle during actual cleavage does not demand an increased oxygen supply. Yet, Matthews (12) has shown that in the absence of oxygen the astral rays disappear. This does not show in any way that the asters use oxygen, it indicates that when the normal oxidative processes of the cell are upset the resulting conditions destroy the asters.

Contrary to Matthews (12) and Osterhout (13), Warburg (21) concluded that the respiration of echinoderm eggs is a function of the cytoplasm, and is independent of nuclear synthesis. Warburg's conclusions have been criticised by Robertson (15); and it is true that the data given by Warburg from experiments with normal cells are insufficient to give a decisive proof of his conclusion. At the same time, his evidence from the effects of narcotics on respiration and on nuclear synthesis, taken in conjunction with the data provided in this paper, support the view that there is no direct association between the rate of oxygen consumption and the amount or activity of the nuclei.

SUMMARY.

The process of cell division in echinoderm eggs has under normal conditions no effect upon the rate of oxygen consumption. If the nucleus plays any direct rôle in the oxidative processes in the cell it does so independently of any particular phase of nuclear activity. The development of the egg is associated with an acceleration in the rate of oxygen consumption.

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NOTE ON THE PENETRATION OF HYDROXYL IONS INTO GELATIN JELLIES

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(*Read 3 March 1924.*)

(With Two Text-figures.)

ONE of the characteristic features of living cells is that they are permeable to ammonia, but relatively impermeable to caustic soda or caustic potash. The usual method of demonstrating this fact is by staining cells with neutral red. This dye is readily taken up by living cells and is at first diffused through the cytoplasm, which thereby acquires a distinct red colour. At a later stage the dye is usually aggregated into irregular granules. When exposed to dilute solutions of ammonia the neutral red in living cells rapidly becomes yellow, indicating the penetration of the alkali. In NaOH or KOH the colour of the dye does not change, however, until the cell begins to show obvious signs of degeneration.

The reason for this difference between the weak and the strong alkalies is not known, but it appears to be associated with the degree of ionisation of the base. It is generally assumed that the cell is impermeable to OH' but is permeable to NH₄OH or to gaseous ammonia. It has further been suggested that this is due to the solubility of ammonia in some fatty constituent of the cell surface. The objections to any theory which postulates a continuous fatty layer on the surface of living cells are of course obvious. Such cells would not be freely permeable to water, nor is this objection overcome by the suggestion that such "lipoids" as lecithin or cholesterol enter into the composition of the membranes. A layer of lecithin made permeable to water is also permeable to crystalloids, and many substances soluble in cholesterol do not penetrate living cells, and vice versa. There are, it is true, certain conditions under which a layer of a fat can be made permeable to water, and yet allow the system to exhibit osmotic properties similar to those of the living cell (see Bütschli (1)), but the purpose of this note is to enquire into the possibility of a differential permeability to ammonia and caustic soda without making use of any fatty substance.

The fact that crystalloids diffuse through protein gels almost as rapidly as through water eliminates, at first sight, the possibility that proteins compose the normal cell surface. This is undoubtedly a difficulty, but it is doubtful whether sufficient attention has been paid to the complicated ionic systems which exist in the cells when making comparisons with non-living systems. The fact that unless certain conditions are observed both ammonia and NaOH penetrate living cells at approximately equal rates is generally overlooked. Newton Harvey (2) showed that the rate of penetration of NaOH is very greatly increased if calcium is absent from the surrounding medium. A considerable wealth of evidence exists to

show that the normal permeability of the cell surface depends upon the presence of divalent cations. It, therefore, seemed of interest to see whether the presence of divalent cations such as magnesium and calcium affected in any way the relative rate of penetration of weak and strong alkalies into a protein jelly such as 5% or 10% gelatin. Such jellies were prepared by dissolving 5 or 10 grams of gelatin in 100 c.c. of the required solution, and adding neutral red as an indicator. Roughly 12 c.c. of the gelatin solution were transferred to each of two tubes which were 1 cm. in diameter and graduated into millimetres. The tubes were held vertically and the solutions allowed to set. To one tube was added 12 c.c. $N/10$ NaOH and to the other 12 c.c. of $N/10$ NH_4OH . From time to time the rate of penetration of each alkali was measured.

It was found that the rate of penetration of the two alkalies into the $M/2$ NaCl jelly was approximately equal. The rate of penetration into the jelly containing

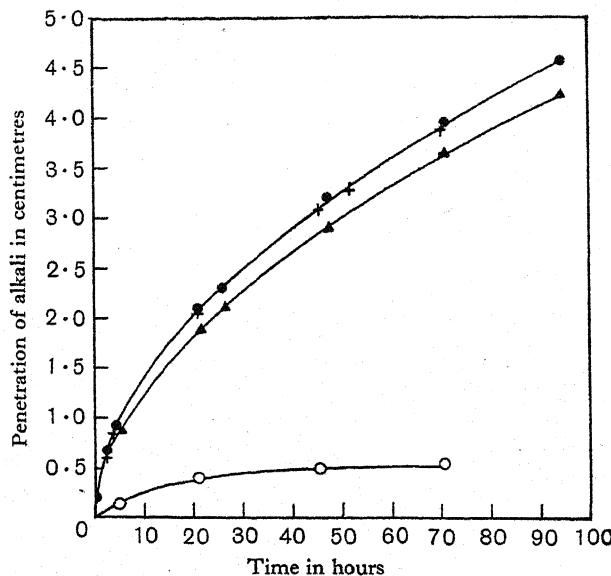


Fig. 1. Graph showing penetration of alkali from $N/10$ NaOH into 5% gelatin jelly containing $M/2$ NaCl (●); $M/2$ $CaCl_2$ + (○); rate of penetration from $N/10$ NH_4OH into jelly containing $M/2$ NaCl (▲).

$MgCl_2$ showed very clearly an inhibiting effect of the magnesium on the penetration of NaOH, whereas it had no effect on the penetration of ammonia.

As the caustic soda penetrates into the jelly containing magnesium, it becomes obvious that an insoluble precipitate—presumably of magnesium hydroxide—is formed within the jelly, and is precipitated in well-marked rings. The position of the last formed ring is always well behind the point reached by the alkali as shown by the change in colour of the neutral red. In the case of ammonia no such precipitate is formed, presumably because the concentration of hydroxyl ions never exceeds that of the solubility product of magnesium hydroxide. The fact remains, however, that a system containing a protein and magnesium is much more readily

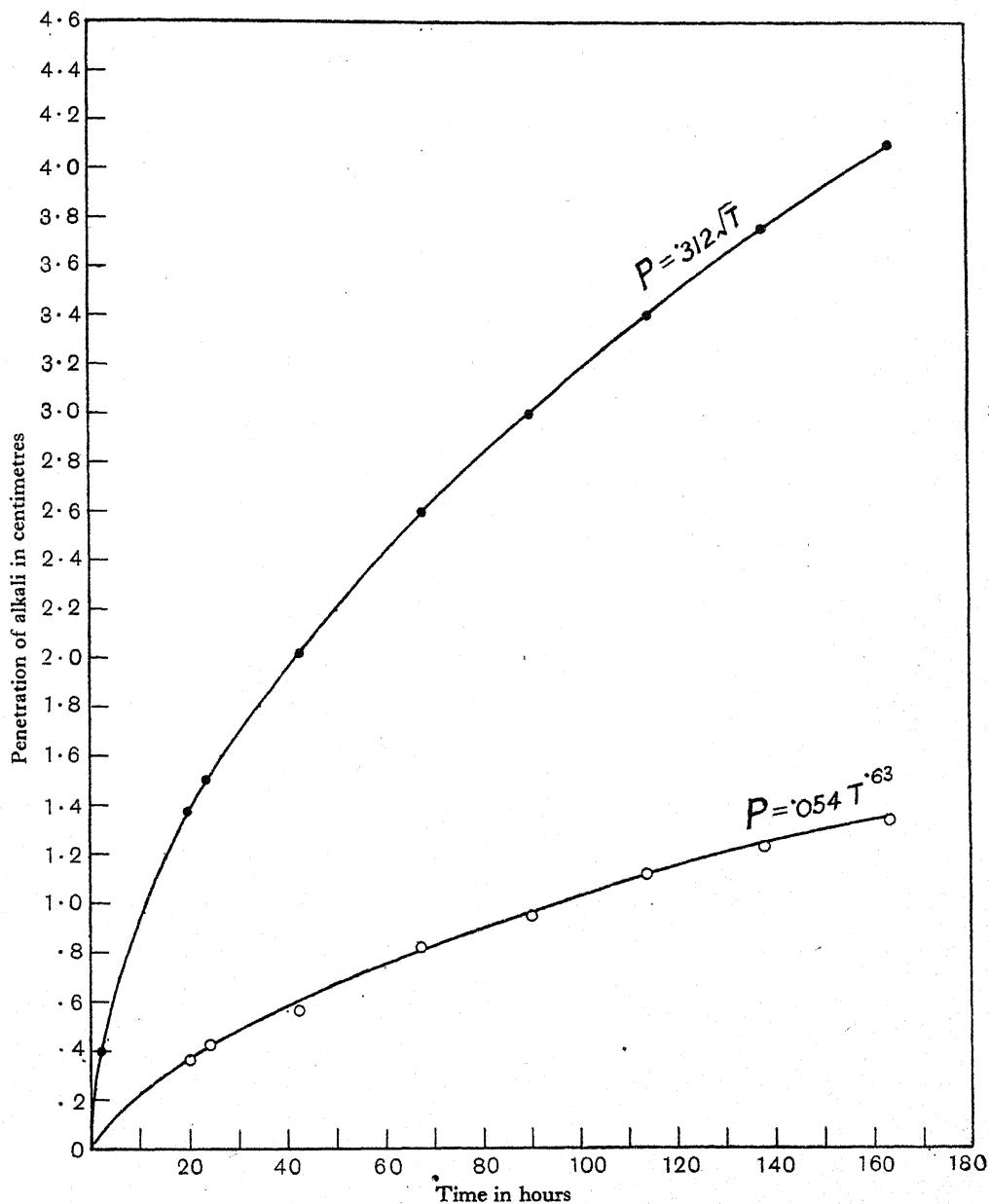


Fig. 2. Graph showing rate of penetration of $N/10 \text{ NaOH}$ (○), and of $N/10 \text{ NH}_4\text{OH}$ (●) into a 10% gelatin jelly containing both NaCl and MgCl_2 (10:1).

permeable to ammonia than to caustic soda, if the penetration of these be measured by the method usually applied to living cells. It will be shown elsewhere that magnesium plays an important rôle in the preservation of the normal permeability of certain cells to hydroxyl ions.

Table I

Time Hrs. Mins.	Distance penetrated in cm. into $M/2$ NaCl jelly (5%) by	
	$N/10$ NaOH	$N/10$ NH_4OH
0 28	.20	.22
3 0	.66	.65
4 46	.90	.87
21 47	2.10	1.93
26 27	2.31	2.13
47 27	3.21	2.92
70 27	3.93	3.63
94 27	4.56	4.22
	$P = .47 \sqrt{T}$	$P = .42 \sqrt{T}$

Table II

Time Hrs. Mins.	Distance penetrated in cm. into 10% jelly in 75 c.c. $M/2$ NaCl + 25 c.c. $M/2$ MgCl_2	
	$N/10$ NaOH	$N/10$ NH_4OH
0 5	.02	.07
0 10	.02	.10
0 20	.02	.17
0 45	.03	.24
1 10	.03	.30
1 30	.04	.34
1 50	.05	.38
2 10	.11	.40
18 5	.34	1.30
20 0	.36	1.37
20 40	.37	1.40
21 35	.37	1.42
22 50	.40	1.47
24 0	.42	1.50
42 50	.56	2.02
68 5	.82	2.60
90 15	.95	2.99
114 25	1.13	3.39
138 35	1.24	3.75
164 0	1.34	4.10
188 40	1.42	4.42
210 45	1.45	4.63
	$P = .054 T^{.63}$	$P = .312 \sqrt{T}$

That the slow penetration of strong alkalies into jellies containing magnesium is due to the deposition of some form of membrane or precipitate is supported by the fact that in the case of calcium no inhibition is found if the concentration of alkali is low enough to prevent the formation of $\text{Ca}(\text{OH})_2$ (Table III). Further

$N/10$ NaOH penetrates equally easily into jellies containing $M/2$ CaCl_2 or $M/2$ NaCl (Table IV).

In the following experiment (Table III) 5 % gelatin jellies were made from the following solutions. A. 90 c.c. $M/2$ NaCl + 10 c.c. distilled water. B. 80 c.c. $M/2$ NaCl + 10 c.c. $M/2$ CaCl_2 + 10 c.c. distilled water. The supernatant alkaline solutions consisted of 90 c.c. $M/2$ NaCl + 10 c.c. $N/10$ NaOH, and 80 c.c. $M/2$ NaCl + 10 c.c. $M/2$ CaCl_2 + 10 c.c. $N/10$ NaOH, respectively.

Table III

Time Hrs. Mins.	Rate of diffusion of $N/100$ NaOH into jelly containing	
	NaCl	NaCl + CaCl_2
1 0	.25	.30
1 40	.31	.38
19 50	1.05	1.05
24 0	1.14	1.17
43 40	1.50	1.50
49 0	1.58	1.60
67 30	1.85	1.83
74 0	1.92	1.92
92 15	2.07	2.10
116 30	2.29	2.30
	$P = .23 \sqrt{T}$	$P = .23 \sqrt{T}$

It is obvious that calcium under these conditions does not inhibit the rate of penetration of strong alkali.

Table IV

Time		Diffusion of $N/10$ NaOH into 5 % gelatin containing $M/2$		
Hrs. Mins.	Hrs.	CaCl_2	MgCl_2	NaCl
1 50	1.833	.47	.05	—
3 0	3.0	.65	.09	.64
4 0	4.0	.81	.12	.80
21 40	21.67	2.08	.39	2.05
26 45	26.75	2.35	.43	2.30
45 30	45.5	3.05	.49	3.05
52 0	52.0	3.25	.51	3.28
70 15	70.25	3.80	.55	3.90
		$.45 \sqrt{T}$	$.17 T^{.27}$	$.45 \sqrt{T}$

This fact indicates that the type of system provided by these jellies is probably not comparable to the surface of living cells. This is supported by other experiments. For example, it can be shown in the case of the cell that all metallic salts inhibit the rate of penetration of alkalies, although the effect of divalent metals is very much greater than that of monovalent metals. In the case of gelatin jellies

alkalies penetrate equally readily in the presence or absence of $M/2$ NaCl. Further if the concentrations of alkalies are reduced to make them comparable with those which penetrate living cells, the inhibiting power of magnesium in the case of gelatin jellies is reduced to almost negligible proportions.

The interest, from a biological point of view, provided by these experiments, lies in the fact that a differential permeability to weak and strong alkalies does not necessarily indicate the presence of a fatty substance. It also suggests the possibility that the protective function of magnesium and calcium in living cells may prove to be due to the formation of some fairly simple membrane or intracellular precipitate.

It may be noticed from the curves that the rate of penetration of alkali follows the general laws of diffusion (see Stiles⁽³⁾). When magnesium is present however the rate of diffusion of hydroxyl ions is no longer a function of the square root of the time.

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ON THE ORIGIN OF THE POTENTIAL DIFFERENCES BETWEEN THE INTERIOR AND EXTERIOR OF CELLS

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VAN SLYKE, WU and MACLEAN (1923) have discussed the equilibria between the plasma and the red blood corpuscles. The principles there laid down can be extended to other cells, and would appear to furnish an adequate explanation, among other things, of the potential difference between the interior and exterior of the cell, which gives rise to the well-known injury current. We shall first consider solutions so dilute that ionization is complete, and the activities of all substances are proportional to their concentrations per unit volume. The cell considered is immersed in an inorganic salt solution, and its wall has the following properties, which that of the mammalian erythrocyte is known to possess:

1. It is permeable to water (Mukai, 1920).
2. It is impermeable to all cations. H^+ and NH_4^+ , which appear to permeate it, are present in very minute quantities, and in these cases it is probable that CO_2 and NH_3 are what really permeate (Gürber, 1895).
3. It is permeable to Cl^- , HCO_3^- ; and other simple anions (Ege, 1922), (Baird, Douglas, Haldane and Priestley, 1923).
4. It is impermeable to colloids, and to certain crystalloidal anions, notably those which contain the organic "acid soluble phosphorus" of the corpuscles.

We will follow Van Slyke and his colleagues in considering for the moment only monovalent inorganic ions.

Let a be the molar concentration of salt outside the cell.

" x " " " " cations inside the cell.

" y " " " " permeating anions inside the cell.

" p " " molarity of proteins and other non-permeating substances in the cell.

" q " " normality of proteins and other indiffusible anions in the cell.

By molarity is meant the weight of indiffusible substances per litre divided by the mean molecular weight of their molecules, each colloidal particle being reckoned as one molecule. By normality is meant the molarity multiplied by the mean negative charge per molecule reckoned in electrons. During life the charge on all, or very nearly all, colloids in the body is negative. Thus in a protein gel probably $p = 0$, at the isoelectric point $q = 0$. Then since water permeates, the osmotic pressure is the same within and without.

$$\therefore x + y + p = 2a.$$

Since the sum of the electric charges in the cell is practically zero,

$$x = y + q.$$

The error in this equation due to the charge of the cell can hardly exceed one part in a thousand, as shown later.

Since the concentration of permeating anions is different on the two sides of the cell wall, the potential difference between them,

$$E = \frac{RT}{F} \log_e \frac{a}{y}, \text{ or at } 38^\circ \text{ C.}$$

$$= 0.017 \log_{10} \frac{a}{y} \text{ volt.}$$

Now

$$x = a - \frac{1}{2}p + \frac{1}{2}q,$$

$$y = a - \frac{1}{2}p - \frac{1}{2}q.$$

Hence the concentration of cations is greater or less in the cell than outside, according as q is greater or less than p . It will generally be greater, as each non-diffusible ion has an average negative charge of more than one electron, not to mention the charge on gels, if present. The concentration of diffusible anions is always less inside the cell, so the interior of the cell is always negative to the exterior.

Since the CO_2 pressure is the same on both sides of the membrane, and the concentrations of HCO_3^- in the ratio $\frac{y}{a}$, while $c\text{H}$ varies as $\frac{[\text{CO}_2]}{[\text{HCO}_3^-]}$ the $c\text{H}$ inside the cell will be to that outside as a to y .

The equilibrium considered differs from the Donnan (1911) equilibrium in that ions of one and only one charge can permeate, and there is therefore a possibility of osmotic equilibrium without a pressure difference.

When we apply these equations to actual cells several corrections must be made, even if we assume that the interior of the cell is a homogeneous sol, which may be nearly true for the red blood corpuscle.

When the solutions are not infinitely dilute, the activities of the various molecular species are nearly proportional to their mol-fractions, on what is practically the same in this case, their concentrations per kilogram of solvent water. If the proteins were largely hydrated the solvent water would be a fraction of the total water, as in the case of strong sugar solutions (Morse, 1914), (Haldane, 1918), but Neuhausen (1922) and Van Slyke, Wu and McLean (1923) have shown that the proteins of plasma and corpuscles only prevent osmotic activity in a small fraction of the total water. The same is probably the case in other cells. Again as there are more polyvalent ions in the cells than outside the ionization within will be less complete than without. The two latter corrections are small, and moreover counteract one another, and we shall be within the limits of experimental error in taking activities as proportional to molar concentrations per kilogram of water. The effects of the CO_2 gradient have also been neglected provisionally. Polyvalent inorganic ions slightly complicate the expressions for x and y given above, but do not affect those for E and $c\text{H}$.

There is reason to believe that most mammalian cells behave like the red blood corpuscles as regards permeability. They are certainly permeable to water. The salts in them must be largely ionized to account for the equality of osmotic pressure within and without them, while the very different concentrations of sodium and potassium in cells and plasma show that the cell boundaries are normally impermeable to them. The only inorganic ions present in large amount are Cl' and HCO_3' . Their permeability to Cl' is shown by its ready replacement by Br' (Nencki and Schumow-Simanowski, 1894). Similarly, Cl' may rapidly replace HCO_3' (Haldane, 1921) in most of the tissues. We may therefore assume that the majority of cell walls are freely permeable to Cl' and HCO_3' , and that the ratio of these ions in them is the same as in plasma and red corpuscles.

In a normal man the ratio $\frac{[\text{Cl}' + \text{HCO}_3']}{[\text{Cl}']}$ is 1.23 (Baird, Douglas, Haldane and Priestley, 1923). Slightly different values in plasma and cells have been obtained by authors using a different technique for bicarbonate estimation, but until a comparison of methods has been made the assumption of free permeability, which in any case is nearly correct, seems simplest.

Now the weight molar concentration of anions in isotonic saline is about 170, so if we know the water and chloride concentrations in any cell the weight molar concentration of $[\text{Cl}' + \text{HCO}_3']$ will be:

$$y = \frac{100}{w} \times 1.23 \times \frac{c}{3.546},$$

where w is the percentage of water in the tissue, c the percentage of chlorine.

$$\begin{aligned} \therefore \log_{10} \frac{a}{y} &= \log_{10} \left[\frac{170 \times 3.546w}{123c} \right] \\ &= \log_{10} w - \log_{10} c - 2.32. \end{aligned}$$

$$\therefore \text{pH of tissue} = \text{pH of medium} - \log_{10} w + \log_{10} c + 2.32.$$

$$\text{Injury potential} = 61.7 [\log_{10} w - \log_{10} c - 2.32].$$

In the table these formulae are applied to a few representative mammalian tissues. Most of the analyses are taken from Abderhalden (1921), that of the erythrocytes and their pH from Van Slyke, Wu and McLean (1923), of the smooth muscle from Constantino (1911). The other pH values are those of Michaelis and Kranzlyk (1914), and the injury potentials those of Nagel (1906). The pH values are calculated on the assumption that the pH of the external salt solution is 7.40. This would be in equilibrium with a cell which was also in equilibrium with a plasma of pH 7.45, the difference being due to the plasma proteins. It will be seen that there is agreement within the very large limits of experimental error. The actual figures obtained are of course averages for many different phases, and have little absolute value. As a means of arriving at the pH of tissues the above method is however probably rather more reliable than a more direct one.

The differences in the anion concentration y in different tissues depends on the values of p and q in them, that is to say on the number of indiffusible molecules and their total charge. In a muscle, for example, the proteins are much farther

from their isoelectric point than the haemoglobin of an erythrocyte, and therefore have probably a larger mean negative charge. Moreover a muscle contains much phosphorus as organic esters which cannot diffuse out. Reckoning this as mainly hexosediphosphoric acid we find a weight molar concentration of $0.021 M$ in rabbit's white muscle (Embden and Adler, 1921). The mean ionic charge on this is probably about 3. Similarly the creatine, which is almost unionized, is about $0.045 M$. Now $y = 0.028$, $\therefore p + q = 2(a - y) = 0.284$. Of this value the hexose diphosphate accounts for 0.084 , the creatine for 0.045 , so that probably the proteins need account for less than half of $p + q$. If the proteins contribute mainly to q this would require a mean negative charge on them of less than one electron per 1200 units of molecular weight, or in other words, less than one equivalent of base bound by 1200 grams of protein.

Table I

Tissue	% H ₂ O	% Cl	pH calc.	pH obs.	P.D. calc.	P.D. obs.	$\frac{y}{a}$
Red blood corpuscles	63.6	0.178	7.17	7.23	14.2	—	.59
Brain (white matter)	70.0	0.155	7.06	—	20.7	17.28	.45
Brain (gray matter)	81.5	0.115	6.87	—	32.7	—	.30
Smooth muscle	80.0	0.112	6.87	—	32.9	—	.29
Liver	84	0.066	6.78	6.40-7.04	38.3	—	.24
Striped muscle	76	0.061	6.62	6.02-6.91	47.8	40-80	.168

We can get a rough idea of the magnitude of the charge on a cell due to the negative potential of its interior by considering the erythrocyte. Its membrane seems to consist in part at least of lecithin and cholesterol, and can hardly be less than two molecules, or about 5×10^{-7} cm. in thickness. Cholesterol has a dielectric constant of 5.4 (Keller, 1921), while that of lecithin is probably not far from 3.1, that of triolein, so we may take that of the membrane as about 4. The capacity of a conducting sphere of diameter d coated with a dielectric of small thickness x and dielectric constant K is $\frac{Kd^2}{x}$. That of a similarly coated disc of the same diameter is $\frac{2Kd^2}{\pi x}$; that of the erythrocyte would be intermediate, say $\frac{3Kd^2}{4x}$. In man $d = 7.5 \times 10^{-4}$. Hence the capacity of an erythrocyte with a bimolecular lipid membrane would be about 3.4 E.S.U. At a potential of 0.014 volt its charge would be 1.6×10^{-4} E.S.U. or 3.3×10^5 electrons. Its volume is about 9×10^{-14} litre and its molarity of $[Cl^- + HCO_3^-] = 0.064$. Hence it contains 5.8×10^{-15} mol, or 3.3×10^9 of these ions. Owing to its charge there is therefore an excess of only one in ten thousand. The average cell would have a larger potential but a smaller capacity per unit volume, and would yield about the same figure. If the membrane contains much water the figure might be reduced nearly twenty-fold, if on the other hand it consists largely of protein it would be somewhat increased. There may of course be an additional charge on cells due to the colloidal properties of their membranes, however a direct determination of the charge on a cell of known composition should enable us to place a lower limit to the thickness of its boundary.

If the cell boundary became permeable to crystalloidal cations as well as anions, though still impermeable to colloids, the cell would tend towards the Donnan (1911) equilibrium, anions, cations and water entering it. It would therefore swell, and would not reach equilibrium unless its wall were rigid or possessed a high surface tension. A cell whose boundary has not one of these properties and is permeable to water *must* therefore be impermeable to ions of at least one charge. On the other hand, it is possible that action currents may be explained by a temporary and local permeability of the cell boundary to cations. The exact potential difference developed in this case would depend on the relative velocities of different ionic species through the boundary, but it would be of the same sign and order of magnitude as the injury potential.

The potential and *pH* differences between the interior and exterior of a cell vary as $\log \frac{a}{y}$, and are therefore increased by the presence in the cell of any non-basic solute which cannot escape from it. When therefore any such substance is synthesized in the cell anions will be extruded and the cell sap will become more acid and electronegative. The acidity cannot rise beyond the isoelectric point of the cell colloids, but where a cell contains large amounts of a negatively charged colloid such as zein or pepsin which is not an ampholyte, very high acidities might be reached. Again if the membrane becomes permeable to any cation this will accumulate in an acid cell, and it is possible that some of the facts of secretion may be explained on these lines.

The theory here developed is largely a simplification of that of Warburg (1922). It differs from that of Bernstein (1913) in not postulating permeability of the cell wall to cations. It agrees in placing the origin of the injury current in the undamaged cell wall, as he showed to be the case by altering the temperature of the undamaged part of the muscle. It agrees with Beutner's (1920) theory in postulating membranes permeable to one ionic species only, but differs in the mechanism postulated, and in calculating the potential of a cell from known chemical data. I have to thank Prof. A. V. Hill for useful criticism, and Mr F. J. W. Roughton for reading the manuscript.

SUMMARY

The observed facts as to the permeability of cells to electrolytes account quantitatively for the injury potentials and hydrogen-ion concentrations of tissues. These can be calculated from their chlorine and water contents.

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THE TRICHOCYSTS OF *PARAMECIUM*

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(With Nine Text-figures.)

IN cultures and in small aquaria *Paramecium* often congregate together in dense masses. These masses are of a whitish colour and are easily visible to the naked eye. They form generally just below the surface and are attached to solid objects immersed in the water. So closely do the *Paramecium* adhere together that portions of these masses may be scraped off the sides of the culture vessel and picked up with a pipette as an almost solid ball of *Paramecium*. On shaking very gently the mass disintegrates, the *Paramecium* separate, and swim freely in the water.

An artificial aggregation on which observations may be more easily made may be produced in the following manner. A glass tube of 3-4 mm. diameter, about 10 cm. long and closed at one end, is first cleaned thoroughly. The tube is now completely filled with culture water containing *Paramecium*. Stand the tube upright and allow it to remain in this position. After an hour or so the *Paramecium* will be seen to have left the surface, where they collected first, and the upper layers to a depth of 2-3 mm. have been deserted. A hollow ring will be observed just below the deserted surface layers. This hollow ring is composed of *Paramecium* adhering to the glass in great numbers. In both this and in the next method recommended for producing aggregations artificially, it is essential that large numbers of *Paramecium* be present in the water in order to produce sufficient contrast between the deserted upper layers and the crowding that takes place immediately below them. If they are not sufficiently concentrated in the culture, they should be concentrated by centrifuging before placing in the tube. Further it is necessary for the concentration of the alkaline carbonates dissolved in the culture water to exceed 0.002 normal and for the pH of the culture water to be less than 8.0. If the pH exceeds this value it may be lowered quite easily by placing 5 c.c. of the water in a test-tube, breathing into the test-tube and shaking. These conditions, however, are nearly always fulfilled in flourishing cultures of *Paramecium* derived from old hay infusions. The necessity for these conditions is explained later in this paper.

Another, slightly more complicated, but equally effective method of producing adherence to the sides of a tube is to take, say, 10 c.c. of the culture water with *Paramecium* in it and to divide it into two portions. Centrifuge the *Paramecium* out of one portion and add them to the other so as to concentrate them. We have now in two test-tubes two 5 c.c. portions of culture water, the one containing *Paramecium*, the other free from them. To each portion add a few drops of cresol red as an indicator. Breathe into the test-tube containing the *Paramecium* and shake,

carbon dioxide is absorbed and the liquid becomes more acid; it is sufficiently acid when the cresol red shows no tinge of red or purple. Very little shaking is necessary, as a rule. Next, the water which has been freed from *Paramecium* must be made more alkaline. It must be shaken vigorously in the test-tube for some time or, better still, fresh air should be aspirated through it. The effect of the shaking or the aspiration of fresh air is to allow the carbon dioxide dissolved in the culture water to be given off, thus rendering the water more alkaline. The proper stage of alkalinity is reached when the cresol red indicator begins to turn purple. Now take a glass tube, 3-4 cm. diameter and 10-20 cm. long. Clean it well and fit the ends with waxed corks so that both ends may be closed. The tube is now filled with the culture water containing the *Paramecium* and is then corked tightly at both ends so as to exclude air bubbles. On standing the tube upright the *Paramecium* soon collect in the upper portion of the tube. As soon as this happens lay the tube horizontal, remove the bottom cork and with a fine pipette remove half the water in the tube from beneath the *Paramecium*. This manoeuvre serves, not only to concentrate the *Paramecium*, but also to remove all the feeble swimmers. Replace the water withdrawn with the water free from *Paramecium* and made alkaline as above. Care must be taken in replacing the water so as to obtain a sharp junction between the two liquids. After the water has been replaced, again cork the tube tightly so as to exclude air bubbles. Then stand the tube upright but reverse the original position so that the *Paramecium* on swimming upwards meet the substituted water. On reaching this water the *Paramecium* stop and very soon it will be observed that just below the junction of the two liquids, which are distinguished by differences in the colour of the indicator, the *Paramecium* have formed a hollow ring adhering to the sides of the glass tube. If the tube be left standing it will be seen that the ring gradually travels upwards, moving at the rate of 2-3 cm. per hour. Many of the *Paramecium* composing these rings can be seen with a hand lens to be quite motionless and adhering to the sides of the tube. As the ring moves upwards the *Paramecium* must detach and re-attach themselves a great number of times.

In order to discover, if possible, what the means of adherence was, I placed some *Paramecium* taken from a dense mass in a culture in a cell formed by a glass ring, 3 mm. deep and 1 cm. in diameter, cemented to the slide and covered with a coverglass. I observed that some of the *Paramecium* adhered to the under surface of the coverglass. As the animals remained in the same position I was able to focus the high power on to them and to see how they adhered. They usually adhere by the anterior end from which a number of fine threads are protruded and attached by their distal ends to the glass (Fig. 1). The region of the body from which they are protruded is only that which is in contact with the glass. While the *Paramecium* is so attached the cilia continue to beat all over the body. As a result of this beating of the cilia it often happens that, if the animal is attached by the anterior end only, it twists round and round. The attaching threads get twisted and the animal breaks away leaving portions of the threads sticking to the coverglass. If, however, the whole of one side of the *Paramecium* is attached to the glass, adherence is much more permanent. In the same cell I observed several small collections of 5-10

Paramecium swimming about, adhering together by these threads which had twisted round them. If these threads are the trichocysts, as I suppose them to be, their function is obviously to enable the *Paramecium* to stick in certain places. They use them in much the same way as the mussel uses its byssus threads, but the attachment is not so permanent. Detachment and re-attachment must be very easy, as I have already pointed out, from the manner in which the adhering rings of *Paramecium* move up the tubes. The length of time which a *Paramecium* will remain attached varies very much. If the *Paramecium* are very crowded the free individuals will jostle against those attached and will often set them free. I endeavoured to observe the time during which one individual remained attached and found that it varied from a few seconds to several minutes. I presume that if the *Paramecium* were left undisturbed and that the surroundings did not alter it would remain attached for an indefinite time.

What causes the trichocyst to be extruded in such a way that the *Paramecium* can adhere to a surface? I believe that the mechanism of extrusion is a very simple one. All that is required is to exert slight pressure on the animal and the semi-liquid material of the trichocyst is at once ejected into the water. Only the very slightest pressure is required, for, when the *Paramecium* are observed in cells, the trichocysts can sometimes be seen to be extruded immediately the *Paramecium* collides with an object. In this case the extrusion of the trichocysts does not occur all over the body but only over that region of the body where the force of the collision exerts pressure, the most usual place being from the anterior end. But a *Paramecium* struck by another will extrude its trichocysts in the region where it is struck and the two move about for a moment or two joined together. Although the mechanism for the extrusion of the trichocyst is simple pressure adhesion by means of the trichocysts does not always follow a collision. In fact the condition of the medium affects adhesion by the trichocysts very greatly.

In order to show that it is simple pressure which causes the extrusion of the trichocysts the centrifuge may be employed. *Paramecium* react negatively to gravity (*i.e.* they swim to the top when placed in a vertical tube filled with their own culture fluid). On placing them in small tubes and centrifuging, if the centrifuge be turned slowly the *Paramecium* accumulate at the ends of the tubes nearest the centre. The speed of the centrifuge should now be increased until the tendency of the *Paramecium* to swim towards the centre is just balanced by the centrifugal force. In this case the *Paramecium* do not accumulate at either end of the tube. It is

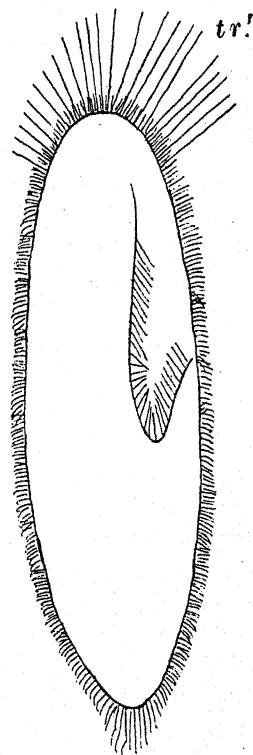


FIG. 1. Outline drawing of a *Paramecium* adhering to the under surface of a coverglass by means of the trichocysts extruded from the anterior end. *Tr.* = discharged trichocysts. (Orig.)

necessary, of course, to use quite short tubes for, at any given speed of rotation, the centrifugal force varies with the distance from the centre. With my centrifuge (a hand one) I found that 26 turns of the handle per minute did not throw the *Paramecium* to the bottom of the tube nor did it permit them to move towards the centre. As the centrifuge was geared 25 to 1 this gives a speed of 650 revolutions per minute of the tubes containing the *Paramecium*. The tubes were 5 cm. long, 4 mm. in diameter, and the closed end was 8.5 cm. from the centre. As the centrifugal force which acts on the *Paramecium* increases with the square of the velocity, it follows that if the handle of the centrifuge be turned 37 times per minute the *Paramecium* will be thrown to the bottom of the tube at a speed equivalent to that with which they swim upwards or centripetally. For if a speed of 26 turns produces a force which just negatives the speed with which they swim upwards, doubling this force will not only negative their upward ascent but force them downwards at a speed similar to that of their ascent under normal conditions. On centrifuging the *Paramecium* for one minute at this speed we find that they are crowded together at the bottom of the tube. On stopping the centrifuge and holding the tube upright nearly all the *Paramecium* composing the mass swim upwards, but a few individuals will be seen to remain sticking to the bottom. On examination with a lens it will be seen that these *Paramecium* are not resting passively on the bottom; their cilia are evidently working vigorously, for they gyrate and sway to and fro, held fast by their invisible bonds. After a short period of straining in this way they break free and swim upwards to join the rest. When the speed of the centrifuge handle is increased to 100 turns per minute so that the speed at which the *Paramecium* are driven downwards is nearly 14 times as great as that with which the animals normally swim upwards, then we observe, after centrifuging for one minute, that the mass of *Paramecium* thrown to the bottom is a very compact one and that on rising they leave behind a gelatinous white mass which corresponds exactly in size and shape with the mass of animals seen immediately after centrifuging. This gelatinous mass does not detain the *Paramecium*. Apart from a few stragglers the bottom of the tube is cleared of *Paramecium* in the same time as when the tube was centrifuged at the lower rate and no such gelatinous mass was produced.

If, with the aid of a fine pipette, we suck up some of this gelatinous mass which remains after the *Paramecium* have left the bottom of the tube and examine it under the high power of the microscope, it will be seen to consist of a number of fine threads crossing and recrossing in all directions (Fig. 2). These threads are formed of extruded trichocyst material. If we centrifuge the *Paramecium* and by

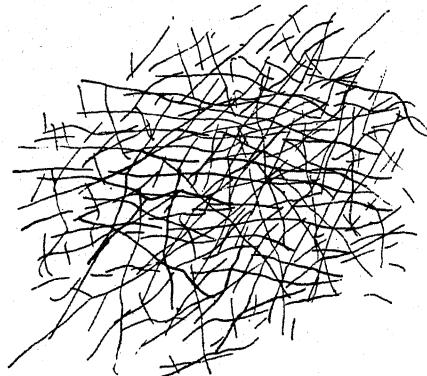


FIG. 2. Intertwining threads of trichocysts composing the residue found at the bottom of a tube on subjecting the *Paramecium* to pressure by centrifuging. (Orig.)

so doing throw them to the bottom of the tubes at speeds which may vary from 4 to 14 times as great as that at which they swim upwards, we find that in every case the gelatinous white mass is left behind when the *Paramecium* rise after the centrifuge is stopped. It is difficult to determine exactly, but it appeared to me that the amount of trichocyst material extruded varied with the speed of the centrifuge, that is, with the pressure exerted on the *Paramecium*.

It is evident that the trichocysts are extruded by pressure. Gentle pressure forces out relatively short straight threads, while greater pressure compels the extrusion of long threads which, intertwining with each other, form a halo of trichocysts round the animal. I am of the opinion that these threads, both short and long, are derived from the same source, from the pocket containing the trichocyst material. They have the same diameter and the same appearance. The long threads are formed by the extrusion of a greater quantity of material. The great difference between the two threads, the short and the long, is that the short ones are obviously means of attachment and serve to hold the *Paramecium* to smooth surfaces while the long threads hardly restrain the animals at all, they easily push their way through the mass of intertwining trichocyst threads and rise from the bottom of the tube after centrifuging. Clearly then the thread itself is not sticky but only the end of the thread. Short threads adhering by their ends and holding the *Paramecium* close to the glass will serve to hold the animal even though the cilia be working, just as a ship in a gale is held fast to the quay by taut hawsers. But if these hawsers lengthen and become slack, then the wind can blow the ship to and fro so that sufficient momentum is very soon acquired to snap the hawsers and the ship is adrift. So it is with *Paramecium*, when the trichocyst threads lengthen then the cilia, which never cease to beat, drive the animal about and the fine bonds are soon snapped.

THE CONDITIONS IN THE MEDIUM CAUSING ADHERENCE.

The conditions under which adherence takes place by means of the trichocysts must now be defined. When *Paramecium* are attracted to a certain spot in such numbers as to form aggregations, adherence usually occurs at this spot, the trichocysts serving to anchor the animals in the attractive region. What constitutes this attraction? By the following experiments it can be shown quite easily that *Paramecium* tends to aggregate in regions of definite *pH* (and that when they do so they adhere), but by varying the conditions (e.g. the concentration of the dissolved salts) a *pH* which formerly attracted now repulses.

Centrifuge 10 c.c. of the water of a flourishing culture of *Paramecium* and with a fine pipette take up the *Paramecium* with as small a quantity of culture fluid as possible and add them to 10 c.c. of Cambridge tap water¹. Divide this tap water into two equal portions and place each portion in a test-tube. By shaking one tube in the air and by breathing into the other and then shaking the *pH* can be adjusted so that it is 8.6 in one tube and 7.0 in the other. Now take a tube similar to that described

¹ The Cambridge tap water is .0042 *N* for alkaline and alkaline earth carbonates (or "excess base" as this is sometimes called).

on pp. 249 and 250 and fill half of it with the tap water and *Paramecium* at pH 7.0 and the other half with the water at 8.6. Cork the tube tightly at both ends and lay it horizontal and at right angles to the source of light. The *Paramecium* accumulate in the middle of the tube on the alkaline side of the junction of the two liquids. If the water has had an indicator, such as cresol red, added to it, it will be seen that the region in which the *Paramecium* accumulate is a narrow band of water of pH 7.8 to 8.0. After a little while large numbers of *Paramecium* will be found adhering to the sides of the tube at this spot. Now prepare another tube, but between the junction of the two tap waters of different pH place some of the clear culture fluid from which the *Paramecium* were centrifuged. Place the tube horizontal as before and lay it at right angles to the light. The *Paramecium* will very quickly accumulate in the culture fluid, leaving the tap water on either side deserted. The pH of the culture fluid when taken direct from the culture is 7.9. We can easily adjust the pH of this culture fluid by shaking it in a test-tube or by breathing into it so that the pH may be any value between 7.5 and 8.5, but, no matter what the pH of the culture fluid, the *Paramecium* will always accumulate in it and will leave the tap water even though the pH of this be adjusted to 7.9, which is the pH preferred when no other choice of water is available. It is obvious, therefore, that the culture water from which they were taken has a supreme attraction for the *Paramecium*. The proper explanation of this preference is very difficult and I have no experimental evidence to support any of the theories that might be propounded.

In aggregations formed naturally it will be observed that the *Paramecium* aggregate and adhere in definite places. They do not, for instance, adhere generally to the sides of the culture vessel or to all the solid objects such as hay stems that may be present. The commonest place for such an aggregation is adhering to some object that lies two or three millimetres below the surface of the water or to strands of alga that may have started to grow in the culture fluid, in exactly the same way as they adhered in the tube described on p. 249.

If we set up a tube similar to that described on p. 249, but before filling the tube add a few drops of cresol red to the culture fluid, it will then be seen that the region just below the surface which is clear of *Paramecium* is more alkaline than the remainder of the culture fluid due to the fact that carbon dioxide is diffusing out of the fluid into the air. Into this region the *Paramecium* do not penetrate but they crowd together just outside it. Evidently they like the pH of their water being raised slightly but not by very much.

It might be objected, following a suggestion of Fox (1920), that the cause of the *Paramecium* not reaching the top is due to repulsion by too high a tension of dissolved oxygen. But that such is not the case here can be shown very easily. By the side of the tube containing *Paramecium* in their own culture fluid set up a similar tube containing *Paramecium* in culture water diluted 20 times with distilled water (i.e. having one-twentieth the concentration of dissolved materials as the original culture). Add cresol red to the liquid before placing it in the tube as before. On standing the two tubes upright and side by side, the *Paramecium* rise to the surface in both tubes. But, on standing, as the carbon dioxide diffuses out

we notice that in the tube containing the natural culture fluid the water at the surface turns purple and that as the purple colour spreads downwards the *Paramecium* retreat from the surface. In the other tube containing culture water diluted 20 times no such change of colour takes place at the surface and the *Paramecium* continue to crowd together at the surface of the water itself, however long we leave the tube to stand. Since the concentration of the dissolved salts in both tubes is too slight to affect the solubility of either the oxygen or the carbon dioxide, we must assume that tension of dissolved oxygen and carbon dioxide will, after standing for a little while, reach the same value in the uppermost layers in both tubes down to a depth of 2-3 mm., for the water in both the tubes is here exposed to the same pressure of oxygen and carbon dioxide, viz. that in the air of the room. Clearly, then, the repulsion from the surface cannot be ascribed to repulsion by too high a tension of dissolved oxygen, nor is it due to too high or too low a tension of dissolved carbon dioxide. The experiment seems to show quite clearly that it is due to *pH*. Proof that this is the correct explanation may be obtained as follows. Take a clean needle and stir the crowd of *Paramecium* which has accumulated at the surface in the tube containing the diluted culture water. Nothing results from this, the animals are evidently not affected by the stirring. Now wet the needle with a little decinormal soda and wipe it almost dry before again stirring the surface. The effect of the soda is seen in the indicator at the surface turning purple down to the depth stirred, which should be no more than 2-3 mm. The *Paramecium* immediately leave this purple layer and behave exactly as they do in the tube filled with natural culture water, concentrating just below the purple layer and forming a ring which adheres to the sides of the tube. The increase in alkalinity in the upper layers brought about by the addition of the soda rapidly diminishes owing to the conversion of the sodium hydroxide into sodium carbonate. The purple colour fades gradually and the depth of the layer diminishes. As the depth of this coloured layer diminishes the ring of *Paramecium* gradually creeps up to the surface again and when the *pH* regains its former value the aggregation no longer consists of a ring of *Paramecium* adhering to the glass but of a crowd in continuous movement just below the surface.

The difference in the *pH* near the surface in the two tubes may be accounted for as follows. The concentration of dissolved alkaline carbonates in the diluted culture fluid as determined by titration with centinormal sulphuric acid using methyl orange as an indicator was found to be 0.00078 normal, in the undiluted fluid it is therefore 0.0156 normal. Such a concentration of alkaline carbonates in the natural culture fluid permits the solution, on exposure to the air of the room, to reach a *pH* of 8.6, whereas the concentration in the diluted culture fluid does not allow the *pH* to exceed 7.5 under the same conditions. Now when the *pH* of the water rises as high as 8.6, the *Paramecium* will be considerably affected, for, when they are placed in water the *pH* of which exceeds 8.2, they cannot rise off the bottom. It is clear that, when the concentration of the alkaline carbonates in the culture fluid is sufficiently great to permit the *pH* to rise above 8.2 at the surface on exposure to the air, then the *Paramecium* will remain below the surface just

below the layer of increased *pH*. When the concentration of the dissolved alkaline carbonates is insufficient to prevent the *pH* at the surface rising above 8.2, then the *Paramecium* rise to the surface itself.

It is important to notice that the *pH* appears to have a very considerable effect on the speed of movement of the *Paramecium*. A culture in which *Paramecium* were adhering freely had a *pH* of 7.95. These *Paramecium*, when taken from the culture observed in the culture fluid in a glass cell, moved comparatively slowly; but, if the *pH* of the water was reduced by the addition of carbon dioxide to the value 7.5 the speed of movement of the animals was most noticeably increased. These same animals, when taken direct from their culture and placed in the culture fluid in upright glass tubes of narrow diameter, did not rise or fall. They did not collect at either end of the tubes and after an hour or two they had formed aggregations which occurred at different places in different tubes. That a water at *pH* 7.95 should cause sluggish movement is important because it explains why the *Paramecium* adhere only when the *pH* reaches a certain value. Below this value the movements are too rapid and the ciliary activity too great for the trichocysts when discharged to be able to hold the animals fast. I suppose that on a collision occurring a small quantity of trichocyst material sufficient to produce a thread about twice as long as a cilium is discharged, but that this thread is not strong enough to hold the *Paramecium* unless the ciliary activity is reduced by the *pH* reaching a certain value. If the *pH* rises above this value then the speed of movement is still further reduced. At this reduced speed the impact of a collision is insufficient to produce the necessary pressure for the extrusion of the trichocysts. The adherent stage is therefore bound to occur at a certain *pH* and will not occur at any *pH* above or below this value. This agrees with the observed facts. I am not prepared to assert that at a certain *pH* all *Paramecium*, from whatever source they are taken and whatever may be the other conditions in the medium, will adhere. But I think it is probable that for every culture there exists a definite *pH*, not far removed from the value 7.9, at which the *Paramecium* will adhere.

In the aquaria and culture vessels the *Paramecium* adhere most freely in old cultures. They adhere as a rule to objects just below the surface or to strands of green algae that may be growing in the water, for the reason, I suppose, that in both these situations the *pH* is increasing slightly. Under favourable circumstances it may be possible to make the *Paramecium* adhere freely by taking them from the culture in their own water and placing them in 5 c.c. of this water in a $\frac{5}{8}$ -in. diameter test-tube and leaving them to stand. On standing the *pH* of the water in the test-tube gradually rises, but there will be no distinct region of more alkaline water just below the surface as in the case of the narrow tubes previously mentioned. Convection currents mix the water and serve to make the increase in alkalinity spread evenly through it. This gradual rise in alkalinity often serves to make many of the *Paramecium* adhere to the sides of the test-tube, where they can be observed with a hand lens. The cultures which I was using were ordinary hay cultures and in them I found that the *pH* of flourishing cultures were all within the values 7.8-8.0. On taking *Paramecium* from such cultures and placing them in test-tubes and

leaving them to stand, I found that as the *pH* rose to 8.0 the animals started to adhere. When the *pH* rose to 8.2 the *Paramecium* were immobilised and fell to the bottom. Exposure to water of *pH* 8.6 kills the *Paramecium*. When *Paramecium* die as a result of the *pH* reaching too high a value no malformations occur, but *Spirostomum* under similar circumstances swells and eventually bursts. Possibly the swelling in *Paramecium* may be prevented by the comparatively rigid ectoplasm with which it is surrounded. From this it is possible to assume that an increase in the *pH* of the medium leads to an increase in the permeability. Water is thus allowed to enter and the pressure so set up in the ectoplasm would favour the easy extrusion of the trichocysts on any additional stress, such as a collision, occurring.

It is curious to note that all the most flourishing cultures had a *pH* of 7.8-8.0. This is, of course, the *pH* value in which *Paramecium* collect when placed in a tube of water showing *pH* gradient of from 7.2-8.6. But it is rather remarkable that it is a value at which ciliary activity begins to decline and that a very slight increase in *pH* above this value results in complete disorganisation. Cultures, the water of which showed a higher *pH* than 8.0, never had any *Paramecium* living in them, although, when the culture had been previously examined and the *pH* was below this value *Paramecium* had occurred. It might, of course, be urged that adherence by means of the trichocysts is a pathological phenomenon due to the *pH* rising too near the lethal value, but this view cannot be held when we consider the fact that adherence only occurs in the most flourishing cultures and at the *pH* value to which the *Paramecium* are attracted when presented with a choice.

The best way that I have discovered of getting the *Paramecium* to adhere and at the same time allow observation under the high power of the microscope is to take the animals from a thick culture in which they are observed to be adhering and place them in a glass cell. Cover the cell with a coverglass after filling it completely full of water. Allow the edges of the coverglass to rest, not directly on the wall of the cell, but on a thin film of water covering the top of this wall. Let this film be adjusted in thickness by withdrawing or adding water so that the *Paramecium* just cannot escape through it. Through this film of water diffusion of gases can take place and as a result at the circumference of the cell just under the coverglass will be produced a circle of water, the *pH* of which very slightly exceeds that of the rest of the water. Many *Paramecium* will swim into this circle and will be seen trying to push outwards into this film. Some may be observed, if we are fortunate, to adhere to the sides of the cell or to the under surface of the coverglass. They remain motionless, but for the movement of their cilia. If we are quick and the *Paramecium* is not jostled away by its fellows we can distinguish that it is held fast by fine threads stretched taut. If a strand of a filamentous alga be stretched across the cell just beneath the coverglass the *Paramecium* will often adhere to this strand and may be observed when so adhering, the reason for this, of course, being that the alga has by its protosynthesis, very slightly increased the *pH* of the water near it.

PREVIOUS VIEWS OF THE STRUCTURE AND
FUNCTION OF TRICHOCYSTS.

I have not been able to discover any observation on the trichocysts of *Paramecium* later than 1914. In reviewing these now rather old observations it must be borne in mind that the authors were concerned primarily with the discovery of structures, so, when the limits of microscopic vision are reached, the theories put forward to account for the reactions of these very small objects are all based on the hypothesis of an invisible structure. The assumption that the trichocysts were weapons of offence and defence, like the nematocysts of the Coelenterata, creates a distinct bias. An amorphous substance was regarded as incapable of behaving as a trichocyst did. The behaviour of the trichocyst, too, was complicated by the use of many different reagents to produce extrusion and the results so obtained were often interpreted with a lively imagination. Verworn's (1889) view, that the trichocyst was a pocket containing a semi-liquid material which hardened on contact with water, was regarded with scepticism, for later researches had shown that the trichocysts, at least before discharge and to a certain extent after discharge, had a constant form. When Mitrophanov (1905) championed Verworn's view his work was so severely criticised that it never gained any general acceptance.

All observers of trichocysts have based some theory of their own as to their mode of action and function on their own observations. These theories usually fit only the facts observed by the author who propounds them, those of other authors often controverting them. I shall try, in reviewing these earlier observations, to strip them of all the theories which have embellished them and to put forward the facts alone. Then I hope to be able to show that the view that the trichocysts are adhesive organs derives support from the observations of all those who have worked at the subject.

There are four papers published in the *Archiv für Protistenkunde* between the years 1903 and 1914 which deal either wholly or in part with the trichocysts of *Paramecium*. Three of these, by Maier (1903), Schuberg (1905) and Khainsky (1911), all agree in their main outlines and it is the views of these three authors that have been accepted by all the text-books. Mitrophanov (1905), the author of the other paper, put forward observations which supported the Verworn (1889) hypothesis. None of these workers pay much regard to the nature of the fixing reagent or its effects and they are generally extremely uncritical. They do not compare the results given by their preparations with the same structures as seen in the living animal, which is surely the only proper criterion by which a fixative can be judged good or bad, but instead they assume that the fixing reagent which they have used is the only one giving the true picture. Moreover, some of Schuberg's figures of the surface of a *Paramecium* as seen under the highest magnification possible present a very suspicious resemblance to Abbe's well-known diffraction images. If, however, we do compare the living animal with the fixed preparations and also take into account the nature of the fixative used and its mode of action we can explain quite easily the divergences in the views of these workers.

Let us first consider Mitrophanov's observations. The important points to notice in this paper are:

(1) That the trichocysts in his preparation are not in every case extruded all over the body, but that they are sometimes extruded only at the anterior end. This method of local extrusion coincides with my observations on the living material.

(2) That the trichocysts lie in the ectoplasm. They are cigar-shaped bodies whose length is equal to the thickness of the ectoplasm. According to Mitrophanov the trichocysts are formed in the region of the nucleus and move later into the ectoplasm. The ectoplasm of the *Paramecium* consist of two layers, the outer and thinner alveolar layer and the inner and thicker cortical layer. The main part of the trichocyst lies in the cortical layer, but a more slender portion passes through the alveolar layer to the exterior.

(3) That the trichocyst is not extruded whole, but a fine thread is drawn out from it. These threads drawn out from the trichocysts are shown by Mitrophanov usually as a single filament, but sometimes they appear to anastomose with other filaments and sometimes the filaments have little blobs on them.

The observations in (2) above are confirmed and agreed on by other workers, while those in (1) and (3) are disputed or contradicted. Copies of Mitrophanov's figures are given in Figs. 3 and 4.

All observers are agreed that the undischarged trichocyst shows a differentiation into two portions and that these two portions differ slightly in their reaction to stains. The outermost portion, which stains darkly, lies in the alveolar layer while the inner and larger portion lying in the cortical layer does not stain so readily. Khainsky's figures show that the discharged trichocyst grows at the expense of the lighter staining material which elongates, while the outer, darker staining, portion is formed into a structure which is generally known as a "head." Khainsky says that the shape of this head is constant, while Schuberg says it varies. (They used different fixatives.) The shape is unimportant, but it is important to note that at the end of the trichocyst there has been observed, after discharge under certain conditions, a "head" which differs from the rest of the material discharged. This "head" is, I believe, the adhesive portion of the trichocyst. It is derived from the outer portion lying before discharge in the alveolar layer while the thread is derived from the inner portion in the cortical layer and may be drawn out to any length from the reservoir of semi-liquid material.

The structure of the discharged trichocyst as figured and described by Schuberg and confirmed by Khainsky is accepted as correct by all the text-books (Fig. 5, a), but I doubt myself whether any such structures are ever discharged by the living

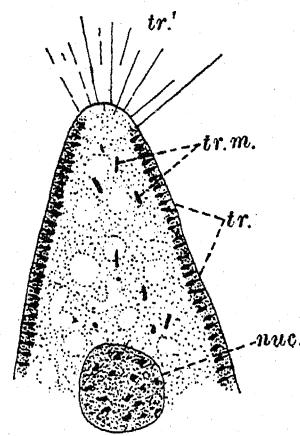


FIG. 3. Section through a *Paramecium* showing the trichocysts discharged at one end and trichocyst material in the endoplasm for replenishing the losses. *tr.* = undischarged trichocysts, *tr.*' = discharged trichocysts, *tr. m.* = trichocyst material, *nuc.* = nucleus. (After Mitrophanov.)

animal. They are sufficiently large to have been observed after discharge from the living animal, being four or five times as long as a cilium and much stouter, yet they have never been seen. Is it not rather strange that they only appear in fixed material and then only when the fixing reagent is a powerful one? Figure 6, which is copied from Khainsky's paper shows the trichocysts in various stages of discharge under the influence of a strong fixing reagent. If the figures of the discharged trichocysts as given by Schuberg and Khainsky are the only correct ones, what is the explanation of the phenomena to be observed when *Paramecium* are treated in the following manner?

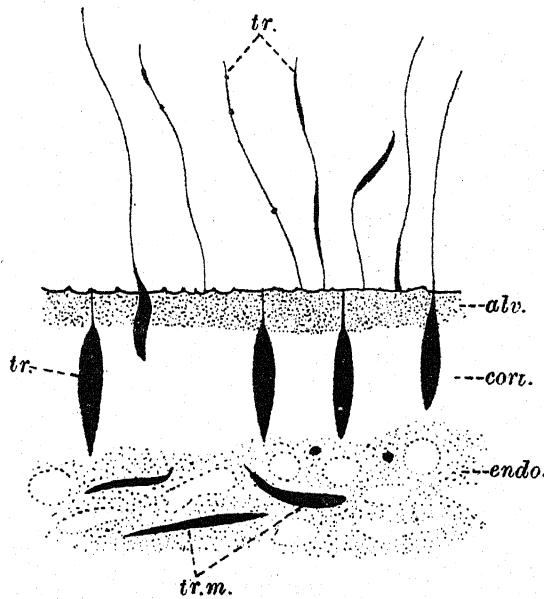


FIG. 4. Section through the edge of a *Paramecium* showing the trichocysts in various stages of discharge, with trichocyst material in the endoplasm available for replenishing the losses. *alv.* = alveolar layer of the ectoplasm, *cort.* = cortical layer of the ectoplasm, other references as in Fig. 3 above. After Mitrophanov.

(1) Fix some *Paramecium* in osmic or neutral formaldehyde vapour in a hanging drop under observation with a $\frac{1}{2}$ -inch oil immersion. As soon as movement ceases the animals can be seen with their cilia fixed in a more or less natural fashion (*i.e.* as observed in the living state, except that they are no longer in motion) and outside these cilia is a halo of discharged trichocysts. This halo of trichocysts is an extremely fragile affair. Any attempt to make a permanent preparation results in its complete separation leaving the *Paramecium* surrounded only by cilia. Iodine in distilled water is the best stain to add in order to show up the cilia. A further effect of the formaldehyde vapour, but not of the osmic, is to cause the extrusion of clear round blobs from the surface of the ectoplasm, thus showing that the effect of the fixing reagent is to alter the permeability of the exterior. Such an alteration of permeability destroys equilibrium and sets up stresses. These stresses are

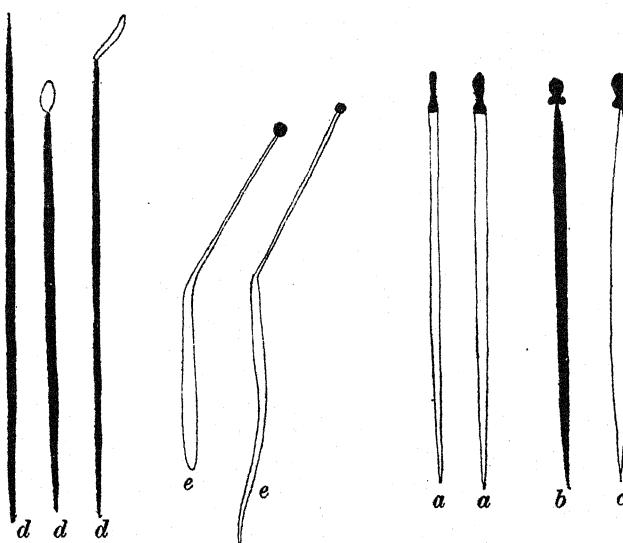


FIG. 5. *a-d*, Discharged trichocysts of *Paramecium* and *Frontonia*, showing the results obtained by the use of a concentrated fixing reagent and subsequent maceration. *a*, *Paramecium* (after Khainsky). Note the variations in the shape of the "head" and its property of staining very readily. *b*, *Paramecium*, and *c*, *Frontonia* (both after Schuberg). The shape of the "head" is regarded as constant by Schuberg, but a difference in the staining reaction of the "head" is shown by him only in the case of *Frontonia*. *d*, *Frontonia* (after Tönniges). The "head" differs from the rest of the trichocyst in its staining reaction, but the shape is not constant. *e*, Trichocysts of *Legendrea* after discharge produced by dilute alcohol (after Penard). The "head" is a round blob, constant in shape and staining very readily.

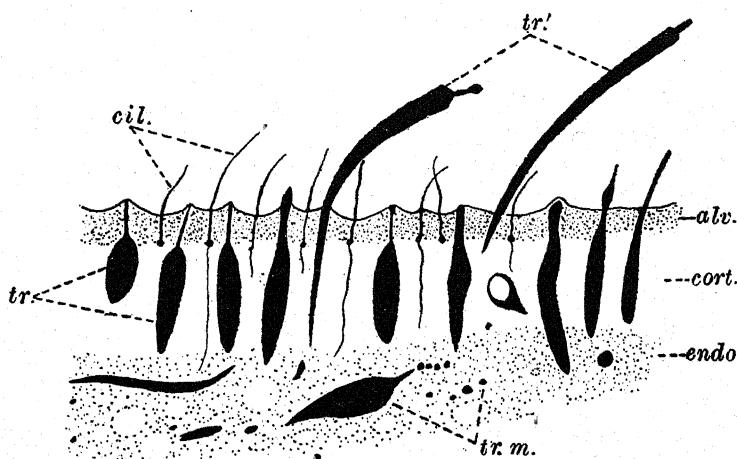


FIG. 6. Section through the edge of a *Paramecium* showing the trichocysts in various stages of discharge, with trichocyst material in the endoplasm for replenishing the losses (after Khainsky). *cil.* = cilia, other references as in Figs. 3 and 4 above. The differences between this Figure and Fig. 4, both of which purport to represent the same phenomenon, can easily be accounted for by the use of fixing reagents of different concentrations.

responsible for the extrusion of the trichocysts. An experiment of Mast's (1909) provides good evidence that stresses in the ectoplasm cause the extrusion of the trichocysts from the region stressed. When *Paramecium* is attacked by *Didinium*, the trichocysts are discharged from the region which surrounds the spot where the fixing organ of *Didinium* is attached (Fig. 7). If the *Paramecium* shakes off the *Didinium* its shape can be seen to have been altered by the local stresses in the ectoplasm set up round the point of attachment of the fixing organ.

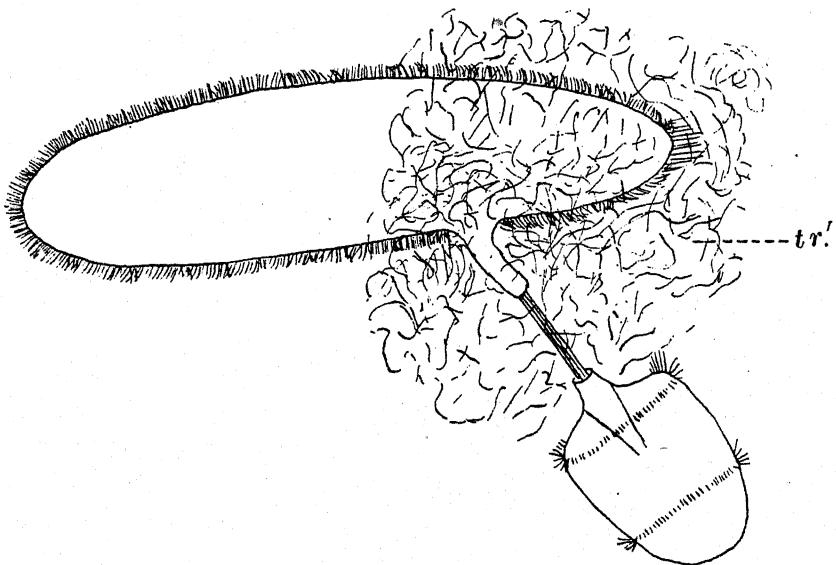


FIG. 7. *Paramecium* attacked by *Didinium* discharging its trichocysts (*tr.*) from the point of attachment. (After Mast.)

(2) Mix as rapidly as possible a drop of culture water containing *Paramecium* with a large excess of either 2% osmic acid or neutral 40% formaldehyde. On staining with iodine the animals are observed preserved in a natural manner. There are no trichocysts visible and these may have been discharged and then separated or they may have remained undischarged.

(3) Mix as rapidly as possible a drop of culture water containing *Paramecium* with corrosive sublimate dissolved in 5% acetic acid or with a mixture of potassium bichromate and osmic acid. Staining with iodine is possible after repeated washings with distilled water. Both these substances are bad preservatives as judged by the appearance of the cilia. In contrast with the reagents used in (1) and (2) above the cilia are lying higgledy-piggledy and they appear to be longer than in the living forms. On examination of a number of *Paramecium* fixed in this way it is very striking how the length of the cilia varies in different animals. This alone makes one suspicious. The "cilia" in this case are nothing more than partially discharged trichocysts, the real cilia being invisible. They have probably all shrunk up. (Khainsky admits that his fixing reagent, which was bromine in water, causes the

cilia to shrink into the body.) If *Paramecium* fixed in this way are teased to pieces it is possible to find structures resembling the discharged trichocysts of Schuberg and Khainsky. If such a fixing reagent distorts the cilia, as is admitted by Khainsky, I see no reason why it should not distort the trichocysts as well. Yet Khainsky takes it for granted that bromine and water will fix a trichocyst properly, although he knows it will distort a cilium.

Both Schuberg and Khainsky disregard entirely the appearance of the trichocysts when discharged as long fine hairs, an appearance which must be familiar to every zoological student. No reasons are given for failing to notice this appearance. It would appear, then, that these discharged trichocysts of Schuberg and Khainsky represent an abnormal appearance due to the fixing reagents used. The enormous stresses set up very suddenly in the ectoplasm by the application of the fixing reagent have submitted the trichocyst material to such pressure that the small opening, through which we may suppose it to be normally discharged with the production of a fine thread, has been forced to open wider with the result that the trichocyst is now foreshortened. Further, the pressure set up is so great as to cause the whole trichocyst, and not a portion of the material composing it, to be discharged. Such a result, although it does not give us a picture of what occurs in the living animal, nevertheless helps us to understand the structure of the fine thread in its natural state. It shows with surprising clearness that the portion first discharged differs from the rest and this accords with the deductions made from the experimental results.

TRICHOCYSTS IN OTHER CILIATES.

The trichocysts in *Frontonia* are very large in comparison with those in *Paramecium* and two observers have studied them in detail. The results of their observations agree with what has already been said about the trichocysts of *Paramecium*. Brodsky (1905) showed that the undischarged trichocysts of *Frontonia* could be separated from the ectoplasm and that when this was done the trichocysts rounded themselves off and then, 10 minutes later, discharged a fine thread. In *Legendrea bellerophon*, studied by Penard (1914), the trichocysts could again be isolated, but in this case they did not discharge spontaneously in water but only when stimulated by dilute alcohol. After isolation in water for longer than $7\frac{1}{2}$ hours the trichocysts failed to discharge on stimulation. These trichocysts discharged after isolation differ from those discharged *in situ* in having no head portion, a fact which is easily verified in *Legendrea bellerophon*, for the head portion is very large and is easily seen in the living animal. Penard concludes from this that the trichocysts are surrounded in the ectoplasm by a very delicate membrane, but, as the discharged trichocysts are different after isolation, this evidence loses much of its value. In *Frontonia* the undischarged trichocyst shows very clearly a distinction into two parts which differ in their reaction to stains. The end of the discharged trichocyst (Fig. 5, d) has an appendix of variable shape and different composition to the main portion (Tönniges, 1914).

Penard's observation on the mode of action of the trichocysts in *Legendrea*

bellerophon are of extreme interest. In *Legendrea* the trichocysts, capable of discharge, are concentrated in batteries at the end of twenty papillae (Fig. 8, *pap.*). Other tricho-

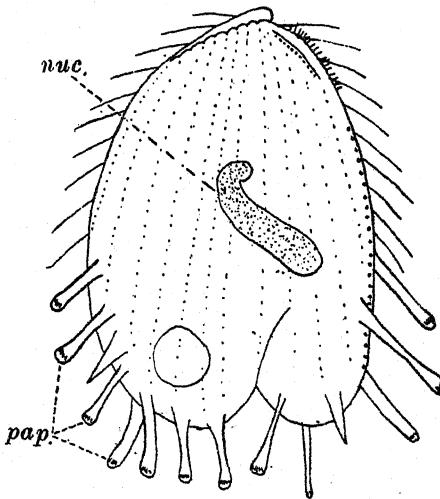


FIG. 8. *Legendrea bellerophon* showing the papillae (*pap.*) on which the trichocysts capable of discharge are borne in batteries. References as in Fig. 3 above. (After Penard.)

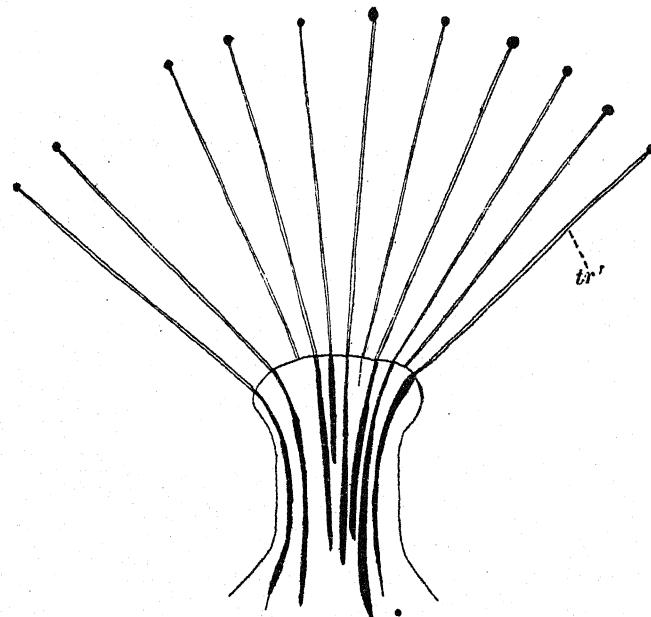


FIG. 9. Enlarged view of a papilla of *Legendrea bellerophon*, showing the trichocysts discharged. References as in Fig. 3 above. (After Penard.)

cysts, incapable of being discharged, can be seen all over the body lying just below the ectoplasm. These trichocysts eventually move up into the papillae and supply

the losses in the batteries occasioned by the discharge of trichocysts here. Penard caused the trichocysts to be discharged by a weak solution of alcohol in which was dissolved some borax carmine. The discharge is extremely rapid, so much so that immediately the alcohol reaches the animal threads appear, which are twice or three times as long as the undischarged trichocyst. At the end of each thread is a round blob which immediately absorbs the carmine and appears bright red. The body of the trichocyst remains in the ectoplasm and is sometimes elongated internally (Fig. 9). This rearward or internal expansion, which is also to be observed in *Frontonia*, indicates that the pressure causing the discharge is a lateral one, such as would be exerted by the ectoplasm on swelling. Discharge is mainly external because, of course, the resistance here is least.

So much for the facts which Penard says he was able to observe over and over again. Then apparently a miracle occurred, the record of which I will give in Penard's own words:

Dans une occasion spéciale même, et pour une raison inexplicable et qui semblerait tenir à une acuité visuelle portée pour un instant très court à une intensité tout anormale, j'ai vu le processus tout entier, qui peut-être n'avait pas duré la dixième partie d'une seconde, se dérouler devant moi comme pendant un temps dix fois plus long: du trichocyste jusque-là inerte partit un large fil, plutôt un tube en apparence, et dont l'extrémité lisse resta nette pendant la course entière; puis tout d'un coup, au moment même où le fil atteignait sa longueur normale ($1\frac{1}{2}$ fois celle du trichocyste primitif), apparut une tête arrondie, dont le diamètre était le triple environ de celui du tube à son extrémité. L'effet, dans ce cas particulier se dessina avec une netteté telle qu'un enfant l'eût décrit de la même façon.

On the basis of this single observation Penard puts forward the view that this blob at the end of the trichocyst (Fig. 5, e) is a drop of poison and that the trichocyst is a hollow tube down which this venomous liquid flows. It is a view that requires the support of more than one observation before it can be accepted. No evidence of *Legendrea* poisoning other animals living with it is brought forward. This view of the function of a trichocyst is as difficult to believe as Tönniges' (1914) view that the appendix at the end of the trichocysts in *Frontonia* represents a primitive trichocyst which these later and larger ones have displaced. Such theories, unsupported by any real evidence, are not worthy of consideration.

THE FUNCTION AND MODE OF ACTION OF THE TRICHOCYSTS.

It is usual to assume that the trichocysts are the weapons of offence and defence of the *Paramecium* and of those other ciliates in which they occur. But the evidence that can be brought forward to support this view is so slight as to raise doubts as to its validity. Doflein (1916) says in the latest edition of his text-book: "Man hat diese Bildungen als Angriffs- und Schutzwaffen gedeutet; doch ist diese Deutung unsicher, und es ist möglich, dass die Ausstossung nur bei starker Reizen infolge Quellungserscheinungen erfolgt." Jennings adopted much the same attitude ten years earlier. The trichocysts look very effective weapons as depicted by Schuberg, whose figures are reproduced in nearly all the text-books, but the pointed end of the trichocyst is behind, the front end being blunt. Apart from this

being an unusual method of constructing a weapon of either offence or defence there is the further difficulty, which I have already pointed out, of their never having been observed in this state in the living animal.

The only recorded instance of the trichocysts serving as a means of defence is recorded by Mast (1909). When a *Paramecium* is attacked by *Didinium*, the *Didinium* fastens itself to the *Paramecium* by a special fixing organ. From the region of the *Paramecium* near the point of attachment of the *Didinium* the trichocysts are extruded very freely (Fig. 7). This mass of trichocyst material is sometimes sufficiently voluminous to force the two animals apart. But the effect is purely mechanical and only occurs when a sufficiently large *Paramecium* is attacked. When a small *Paramecium* is attacked the trichocysts are discharged as before but no effect is produced on the attacking *Didinium*. In *Nassula*, which is a comparatively large Ciliate, the trichocysts apparently function in the same way as they do in the larger *Paramecium* and ward off the attacks of the *Didinium*. But this evidence in favour of the view that the trichocysts are weapons of defence is negatived by another statement in the same paper. Mast says: "On May 1 a specimen of *Frontonia leucas* (?)* was put into a drop of water on a slide containing numerous *Didinium* which had not fed during the preceding two days. This specimen was attacked 58 times before it finally died, 40-45 minutes after it was first put in." Now *Frontonia* is well supplied with large trichocysts but they seem to be quite useless in resisting the attacks of the *Didinium*. In *Frontonia*, of course, the trichocyst when discharged is comparatively short and thick and does not result in the production of a mass of fine intertwining threads as in *Paramecium* and *Nassula*. The evidence of Mast in favour of the trichocysts being weapons of defence is too contradictory. Besides, the fact that the trichocysts function as organs of defence only when the animal reaches a certain size and are present but useless when this size is not attained rather suggests that the real function is not that of defence but that their acting in this way is accidental.

Penard, as already noted, has suggested that the end of the trichocyst is poisonous, but neither he nor any author has produced any evidence to support this view. It remains therefore a pure speculation.

The distribution of the trichocysts among the Ciliata does not give any indication of what their function might be. With the exception of *Frontonia* and *Strombidium*, trichocysts are only found in the *Holotricha*, but they are by no means universally present even here. They have been recorded so far as I have been able to discover from a survey of the literature, in addition to those forms already mentioned, in *Ophryoglena*, in *Dileptus*, in *Loxophyllum*, in *Lionotus*, where they are arranged in a band, in *Prorodon*, where they are present only at the anterior end, and in *Amphileptina*, where they are present on the ventral surface only; while they are absent in *Chilodon*, *Coleps*, *Glaucoma*, *Opalina*, *Stentor*, *Spirostomum*, *Trachelius* and *Colpidium*. In fact they are relatively uncommon structures. Unfortunately we know next to nothing about the habits and life-history of these forms, so that it is impossible to

* I take it that the (?) refers to the specific identification. Mast could hardly have failed to identify the genus.

put forward any views as to why trichocysts should be present in some and absent in others of the same group.

So far as I am aware, I am the first person to observe the discharge of the trichocysts in the living *Paramecium* in its normal environment and without its being stimulated by any reagents. That such an observation when made should give a clear idea of the function is not surprising. This new discovery, that the trichocysts provide a means whereby the *Paramecium* can attach themselves to objects in the water, clears up many of the difficulties of previous workers. That the natural discharge of the trichocysts should closely resemble the discharge when produced by reagents is as might be expected. The chief difference between them is that the discharge produced by reagents usually affects the trichocysts all over the body, whereas in the natural discharge it is regional. The only "reagent" which will cause a regional discharge of the trichocysts is the electric current. Ludloff, quoted by Verworn (1915), has shown that an electric current causes a violent contraction of the end of *Paramecium* which lies towards the anode. This contraction not only expels the trichocysts but alters the shape of the end of the body. Another difference between the natural discharge and that produced by reagents is that the latter is more profuse, but this is no doubt due to the difficulty of regulating the artificial stimulus within sufficiently fine limits. In the natural discharge the length of trichocyst extruded is comparatively short, often not much longer than a cilium, but I have never observed any difference between the thickness of such a short trichocyst and that of the long threads produced when a reagent is applied, nor have I ever seen them as described by Schuberg and Khainsky.

I am inclined to think that, in nature where the discharged trichocysts are comparatively short, only the portion of trichocyst material lying in the alveolar layer is used in the discharge. This is the portion which forms the head of Schuberg and Khainsky's figures and is probably, as has been shown by the experimental evidence, sticky. The discharge of great quantities of trichocyst material is useless so far as it helps the animal to adhere and it is produced only by abnormal stresses. After the "head" portion has been discharged as a short thread, the reservoir in the cortical layer pushes fresh trichocyst material outwards into the alveolar layer. Here, I would suggest, this material is slightly altered, the conditions in the alveolar layer making it sticky. This hypothesis makes it possible to explain how a *Paramecium* can so easily detach and reattach itself again by means of the trichocysts. If attachment were to involve the loss of whole trichocysts instead of a minute portion of trichocyst material, replacement of whole trichocysts must occur with considerable rapidity and in great profusion. Although both Maier and Mitrophanov have shown that both trichocysts and trichocyst material occur in the endoplasm in *Paramecium* there is no evidence to show that trichocysts are replaced in a wholesale manner. The replacement possible from this source is no more than would make good the losses occurring from attachment and reattachment taking place by the discharge of small quantities of trichocyst material at a time.

I have shown that the discovery that the trichocysts of *Paramecium* function as organs of adhesion can be supported by the observations of all previous workers

and they no doubt would have interpreted their results as I have done had they been aware of their true function. The difficulties and the discordant views of earlier workers are due entirely to a misinterpretation of the function of the trichocysts. The objection may perhaps be raised that, if the trichocysts are organs of adhesion, why has this fact so long escaped observation. I would point out that the Protozoa are seldom or never examined in their natural environment *sensu stricto*. The conditions of the environment render this wellnigh impossible. They are taken from their environment by rather violent means (violent, that is, so far as the Protozoa are concerned) and examined in a drop of water on a slide. Following this examination three assumptions are made: (1) that the effect of shock on the behaviour of the Protozoa due to removal to the slide can be neglected, (2) that if the drop of water in which the Protozoa are examined is taken from the pond, aquarium or culture in which they may be living, the conditions in the drop will be so little different from the natural conditions that the variation can be neglected, and (3) that the behaviour of the Protozoa in the circumscribed environment of the drop is the same as that in their natural environment. None of these assumptions are justifiable and the most important of them (2) can be directly disproved. Set up a dozen tubes similar to that described on pp. 249 and 250, containing *Paramecium* in their own culture water taken direct from the culture. Cork the tubes at both ends so as to exclude air bubbles and stand them upright. The *Paramecium* very soon accumulate in the upper portion of the tubes. At intervals of 45 minutes take two of these tubes. Withdraw the water from the lower portion of one of the tubes with a clean dry pipette and replace it immediately, exposing it to the air as little as possible. Then reverse both the tubes and the *Paramecium* will swim upwards again. At first no difference will be noticed between the behaviour of the *Paramecium* in the two tubes. But, after 1½ to 3 hours' standing, the *Paramecium* will now, on swimming upwards after the tubes have been reversed, be checked in their ascent when they meet the water which was withdrawn and replaced. The check will be only a slight one at first but it develops so that, in tubes that have been standing for several hours, the effect of withdrawing and replacing the water in the lower portion of the tube, no matter how carefully this is done, is always to prevent the *Paramecium* from rising to the top of the tube when this is reversed, the animals crowding together just below the replaced water but refusing to enter it. In the control tubes the *Paramecium* rise to the top without any check when the tubes are reversed. This experiment shows how extraordinarily sensitive *Paramecium* is to conditions in the medium. It shows, moreover, that this sensitivity is destroyed by the shock of transferring the animals to the tubes and that it is restored on allowing them to stand. Hence the behaviour of *Paramecium* in its natural environment, when deduced from observations made on the animal in a drop of water on a slide, may give an entirely erroneous view. Because it is so extremely difficult to produce and regulate the necessary conditions in a drop of water on a slide or even in a larger vessel, and because the necessity for so regulating the conditions has not been previously recognised, the real function of the trichocysts of *Paramecium* has hitherto escaped observation.

SUMMARY.

1. The trichocysts are the means whereby *Paramecium* adheres to surfaces. Adherence takes place when *Paramecium* are attracted to a particular spot. The cause of this attraction is largely the *pH* of the water, but it may be masked in certain circumstances.

2. The cause of the extrusion of the trichocysts is shown to be slight pressure such as may be set up by the *Paramecium* colliding with an object in the water. Verworn's view that the trichocyst consists of semi-liquid material which hardens on being extruded into the water, is here adopted. It can be shown from the experimental evidence that the tip of the trichocyst thread is sticky, but the rest of the thread is not.

3. Ciliary motion does not cease when *Paramecium* is attached by the trichocysts. The speed of movement, which is dependent on ciliary activity, is reduced when the *pH* of the water in which the animals are living reaches 8.0. This reduction in ciliary activity results in the slender trichocysts being able to hold the *Paramecium* fast, when the *pH* is within 0.1 of 7.9. A further increase in the *pH* of the water above 8.0 results in the speed of the *Paramecium* being so much reduced as to render the force of a collision insufficient to expel the trichocyst. For the *Paramecium* studied the critical *pH* for adherence was 7.95.

4. It is shown that the observations of previous workers on the structure of the trichocyst accord very well with their functioning as organs of adherence.

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A full bibliography of the literature prior to 1900 will be found in the paper by Tönniges (1914).

SPERMATOGENESIS OF *LITHOBIUS FORFICATUS*

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(With Plates XIV-XV and One Text-figure.)

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1. INTRODUCTION.

THE spermatogenesis of *Lithobius* has been the subject of investigation by a large number of workers (1, 3, 5, 7). But all previous workers have used fixatives containing acetic acid and have confined their attention principally to the chromosomes and the mitotic figure. To the best of my knowledge no work has so far been done on the cytoplasmic inclusions by the more recent cytological methods.

In the present paper an attempt has been made to work out in detail the remarkable Golgi elements and the mitochondria in the giant egg-like spermatocytes and their ultimate fate in spermatogenesis. Special attention has been paid to the development of the acrosome which is a prominent structure in the ripe sperm.

The problem of fixation of the testis of *Lithobius* is a very difficult one on account of the presence of unique cytoplasmic fibres which at times attain huge dimensions in the spermatocytes. These fibres are almost completely destroyed by fixatives containing acetic acid as is quite clear from control preparations (Gilson, Bouin and Flemming). Hence none of the earlier workers has figured these extraordinary fibres because all of them have worked with acetic acid.

The study of cytoplasmic inclusions, therefore, becomes a formidable task, inasmuch as these fibres are perfectly preserved in Mann-Kopsch and chrome-osmium and cause a folding of the tissue on itself in the process of section-cutting and thereby causing great distortion. While such a distortion does not at all interfere with the study of the remarkable Golgi elements, it makes the study of the granular mitochondria an almost impossible task. Experiments with the centrifuge were, therefore, performed, with the result that these fibres were thrown on to one side of the spermatocyte, while the mitochondria and the Golgi elements remained

uniformly scattered. The study of the granular mitochondria was thus made possible. The testes were dissected out of the animals and centrifuged in normal salt solution for half an hour by a hand centrifuge. They were then thrown into capsules containing various fixatives. A safer method, however, is to centrifuge the live animal and then to dissect out the testis. The dissection takes only half a minute.

Mann-Kopsch and Champy's fluid followed by iron haematoxylin and Benda's alizarin and crystal violet were used. A large number of experiments were performed with Da Fano's silver precipitation method, but, although the spermatocytes and the fully-ripe sperms were satisfactorily fixed, the general fixation of the testis was very unsatisfactory. The formalin-cobalt nitrate mixture of Da Fano does not seem to be suitable for this peculiar tissue.

I have to thank Mr J. Gray, M.A., Fellow of King's College, under whose supervision this work has been carried out, for his valuable advice and also for correcting the manuscript of this paper.

2. SPERMATOGONIUM AND SPERMATOCYTE.

Fig. 3 shows a centrifuged primary spermatocyte fixed with Mann-Kopsch. At *CF* are the peculiar cytoplasmic fibres which have been thrown at one end of the egg-like spermatocyte. Generally these fibres are blackened with osmic acid. In many cases the area of cytoplasmic fibres may be so intensely blackened with osmic acid that it is impossible to decolorise it in turpentine. The mitochondria at *M* and the Golgi rods at *GA* remain uniformly scattered throughout the cytoplasm. In unstained Mann-Kopsch preparations the granular mitochondria appear as clear, straw-coloured bodies, but in many cases it is difficult to see them in the region of the fibres, if the latter are closely grouped together. They can be easily stained a light pink colour with Altman's acid fuchsin. The Golgi elements appear as intensely black curved rods. Each rod may be associated with a clear, circular or semi-circular archoplasm which appears brownish in unstained sections. As a result of centrifugal force the two ends of some of the highly-curved rods may approximate and result in ring-like elements. The nucleus remains in the centre.

When a centrifuged spermatocyte is treated with Benda, the fibres stain very lightly with alizarin and the mitochondria take a light violet stain. The Golgi elements appear as beautiful deep violet rods and the archoplasm is red. With Champy followed by iron haematoxylin the fibres appear light grey or quite colourless according to differentiation. The mitochondria stain grey and the Golgi rods are black.

In successful Da Fano preparations the fibrous region is grey or yellow, but generally grey, the mitochondria are yellow or deep golden and the Golgi rods are black.

When a centrifuged spermatocyte is treated with fixatives containing acetic acid (Fig. 5, Gilson), the fibres are extremely distorted and give rise to a sort of trabecular area, a portion of which may sometimes be removed from the spermatocyte in the process of sectioning. The mitochondria appear as distorted granules.

The Golgi elements are never completely destroyed by acetic acid and appear as irregular bodies of various sizes which stain deeply with iron haematoxylin. As will be shown later the "metaplasma" of Blackman⁽¹⁾ and Meves and Von Korff⁽⁵⁾, or the "formations ergastoplasmiques" of Bouin brothers⁽³⁾ are really the remarkable Golgi elements which, though highly distorted by acetic acid, are not completely destroyed by it.

The amount of Golgi rods in a spermatocyte varies considerably. In a few cases the amount is so large that the whole cytoplasm is full of them. When such a spermatocyte is centrifuged (Fig. 4, Mann-Kopsch), the cytoplasmic fibres seem to be entangled amongst the Golgi elements and are not thrown at one end of the spermatocyte. Most of the Golgi elements in Fig. 4 appear as rings which undoubtedly result from the approximation of the two ends of the rods caused by centrifugal force and also by overcrowding.

When an uncentrifuged Mann-Kopsch preparation (Fig. 2) is mounted unstained the whole cytoplasm appears quite black. This is due to the peculiar cytoplasmic fibres becoming quite black with osmic acid. If, however, the slide is kept in turpentine for seven days, the thin fibres give up the black stain, but the thick ones and the Golgi rods still remain black. The mitochondria appear as granular brownish bodies.

From an account given above of the extraordinary cytoplasmic fibres of the spermatocytes of *Lithobius*, which at times attain huge dimensions, it will be clear that they are destroyed to a large extent by acetic acid and are best studied with fixatives without acetic acid. Of these Mann-Kopsch is the best. Thus it is that none of the earlier workers on *Lithobius* has figured them. To the best of my knowledge such fibres have been described in one other chilopod only, namely, *Scolopendra morsitans*, by Prenant⁽⁷⁾ in the year 1886. He figures fibres similar to those shown in my Fig. 2 in those preparations only in which he used osmic acid alone, whereas in his acetic acid preparations they are not figured.

Although the mitochondria and the Golgi elements remain uniformly scattered in the centrifuged spermatocyte of *Lithobius*, they behave differently in the centrifuged egg (Nath⁽⁶⁾). In the latter the granular mitochondria and the Golgi rods occupy a central position. This is due to the fact that the heavy yolk associated in some way with nucleolar extrusions is thrown down and the light Golgi yolk goes to the upper pole.

The spermatogonia (Fig. 1, Benda) are comparatively small spindle-shaped cells lying at the periphery of the testis. Each spermatogonium has a few Golgi rods and granular mitochondria, but the extraordinary fibres present in the spermatocytes are absent. Probably the fibres are very thin and become prominent only as the growth of the spermatogonium proceeds.

3. THE DISTRIBUTION OF MITOCHONDRIA AND GOLGI ELEMENTS DURING MEIOSIS.

So far as I have been able to ascertain, the mitochondria and the Golgi elements are distributed passively in roughly equal parts to the two daughter cells in meiosis.

Fig. 6 (Mann-Kopsch) shows the metaphase of the second meiotic division. The individual rays of the mitotic figure cannot be seen in such a preparation, but the ray area is very clearly defined. The chromosomes appear as round brownish bodies. The mitochondria and the Golgi rods are uniformly distributed and are sorted out into two sub-equal parts to the two resulting spermatids (Figs. 7 and 8).

4. SPERMATELEOSIS.

(a) THE ACROSOME AND GOLGI ELEMENTS.

Fig. 7 (Mann-Kopsch) shows a newly-formed spermatid. The nucleus lies in the centre of the cell and the Golgi elements are uniformly distributed. An important change has now come over the elements. Most of them assume the form of rings instead of rods. These rings can be demonstrated most beautifully with Benda. With Mann-Kopsch each ring may become so black that it may appear as a solid circular body.

As transformation of the spermatid proceeds, the nucleus leaves the centre and comes to lie in the anterior part of the cell. The majority of the Golgi elements come to lie at the anterior end of the nucleus (Fig. 8, Mann-Kopsch). This, however, is only a temporary condition. Sooner or later most of the elements go into the tail region except a few which remain near the anterior face of the nucleus (Fig. 9, Benda). Fig. 10 (Mann-Kopsch) shows a transverse section of the tails of sperms of about the same stage as shown in Fig. 9.

As attenuation of the sperm proceeds the Golgi elements which were lying near the anterior face of the nucleus get stuck to the nuclear membrane and considerably shrink in size (Fig. 11, Mann-Kopsch). Since they ultimately give rise to the acrosome, they will henceforward be called the acroblasts. The acroblasts, like the Golgi elements in the tail region, continue to shrink in size (Fig. 12, Mann-Kopsch). In Figs. 13 and 14 (Mann-Kopsch) at *A* is the cap-like acrosome which is probably secreted by the acroblasts. It goes black in Mann-Kopsch preparations. In each of the above two figures is another nucleus whose anterior face is stuck to the cell membrane, thus obscuring the acrosome.

On account of the extreme attenuation of the sperms after the stage represented in Fig. 14 and the very complicated coiling of the tails and the nuclei, both of which rapidly elongate, I have found it impossible to study the further development of the acrosome in sections. In the fully-ripe sperm, however, the acrosome has been studied by a special method to be described later.

The acrosome stains black and violet in iron haematoxylin and Benda respectively.

(b) MITOCHONDRIA.

Up to the stage represented in Fig. 11 the mitochondria are uniformly distributed. After this stage, however, all the mitochondria go into the tail region and like the Golgi elements form the sheath of the axial filament. The region immediately round the nucleus is quite free from the mitochondria (Figs. 12, 13 and 14).

(c) THE AXIAL FILAMENT.

Although the axial filament develops as early as the stage represented in Fig. 9 (Benda), in Mann-Kopsch preparations it can be seen in much later stages only (Figs. 13 and 14). It stains deeply with crystal violet and iron haematoxylin. Like the coiling tails of the maturing sperms it has a sinuous course, as is clear from Fig. 9, where it has been cut in two different places.

The axial filament can be seen very clearly in the fully-ripe sperm. Fig. 15 (Mann-Kopsch) is a transverse section passing through the tails of the ripe sperms. The axial filament appears as a very distinct dark dot in the centre of a small circle which is the tail sheath. The latter is directly formed from the mitochondria and the Golgi elements.

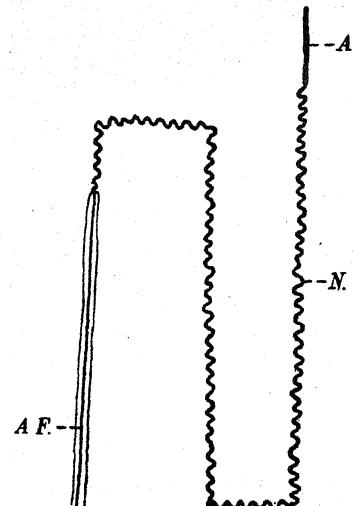
(d) THE BASAL GRANULES.

Certain small granules constantly occur at that portion of the nuclear membrane of the sperm from which the axial filament starts (Figs. 13 and 14). The peculiarities of these granules are that they become intensely black with osmic acid and are stained black and violet with iron haematoxylin and crystal violet respectively. At an early stage during spermatogenesis a few Golgi elements get stuck to the posterior face of the nuclear membrane in the same way as the acroblasts get stuck to the anterior face. They progressively shrink in size and probably give rise to the basal granules (Figs. 9, 11, 12, 13 and 14).

(e) THE RIPE SPERM.

Text-fig. 1 is a Da Fano preparation of the ripe sperm. At *A* is the prominent, straight acrosome; at *N* is the very long thin screw-like nucleus, and at *T* is only a small portion of the thicker tail. The tail is hundreds of micra in length and many times longer than the nucleus. The extraordinary length of the ripe sperm, as well as the extremely complicated way in which the sperms coil within the cyst, will at once make it clear that it is impossible to see in one section a single sperm along its entire length.

Sections being thus useless for the study of the ripe sperms, a method had to be devised by which a single sperm could be studied along its entire length. If a ripe testis is cut into small bits, long filaments of ripe sperms hang out from them. They are then fixed either in Da Fano or Mann-Kopsch, and after dehydrating and clearing, they are as gently as possible spread out on a slide in clove oil. The oil is then wiped away and the preparation mounted in Canada Balsam.



Text Fig. 1.
A.=Acrosome. *N*.=Nucleus.
A.F.=Axial filament.

In satisfactory mounts one finds a bundle of thin long screw-like threads which pass backwards into quite straight and thicker threads. The screw-like threads are the nuclei and the thicker threads are the tails which, on focussing, may show a distinct axial filament. (See transverse sections of tails in Fig. 15.)

It is useful here to compare the acrosome of *Lithobius* with that of *Scolopendra heros* (Blackman), which, to the best of my knowledge, is the only other chilopod in which the acrosome has been studied. Although unfortunately Blackman used acetic acid in his preparations, he seems to have given a correct interpretation of *Scolopendra* spermatogenesis which is, on the whole, similar to that of *Lithobius*.

The acrosome in *Scolopendra* is a long structure (much longer than that of *Lithobius*) in front of the still-longer spiral nucleus. It is formed by the union of "Archoplasmic spheres" (probably the Golgi rings which are not completely destroyed by acetic acid) and becomes a big "spathulate" structure in a stage as early as that represented in my Fig. 11. In *Lithobius* the nucleus moves forward (Fig. 13) with the result that there is a small amount of space between the anterior face of the nucleus and the cell-membrane, which is occupied by the small acrosome. In *Scolopendra*, however, the nucleus never moves forward so much and consequently there is a large amount of space occupied by the very prominent "Spathulate" acrosome which becomes very long in the fully-ripe sperm.

At the end of this section it must be mentioned that the whole process of spermatogenesis in *Lithobius* must be likened to an egg with a strong membrane becoming extremely attenuated with the result that no portion of the spermatid cytoplasm is cast off, as is usual in other cases.

5. "METAPLASM" OR "FORMATIONS ERGASTOPLASMIQUES."

Meves and Von Korff⁽⁵⁾, working on *Lithobius forficatus*, have described in the spermatocytes certain irregular densely-staining bodies. Bouin brothers⁽³⁾, working on the same species, also describe such bodies which they have termed as "formations ergastoplasmiques." In the American species of *Lithobius* Blackman⁽¹⁾ also reports the presence of "a few small, distinct granules" which he considers as "metaplasma," that is, food material or by-products of metabolism. Such bodies are also described by Medes⁽⁵⁾ in *Scutigera*. It must be remembered that all these workers used fixatives containing strong acetic acid.

It may be said at once that in my Mann-Kopsch or Benda or Champy-iron haematoxylin preparations no such bodies appear. In my control acetic acid preparations, however, irregular bodies staining densely with haematoxylin and uniformly scattered do appear and these are undoubtedly the Golgi elements which, although very much distorted by acetic acid, are not completely destroyed by it. The Golgi elements undergo the greatest distortion in Gilson, less in Bouin and the least in Flemming. It is impossible to destroy them completely. Fig. 5 shows a centrifuged spermatocyte fixed with Gilson for 48 hours and the Golgi elements, although greatly distorted, are nevertheless present.

6. SUMMARY.

1. The spermatogonium is a somewhat spindle-shaped cell with granular mitochondria and a few curved Golgi rods.
2. During the growth phase a spermatogonium enlarges into a giant egg-like spermatocyte.
3. Each spermatocyte contains granular mitochondria, a large number of highly curved Golgi elements and curious cytoplasmic fibres which at times attain huge dimensions. Each Golgi rod may have a distinct archoplasmic sphere associated with it.
4. In a centrifuged spermatocyte the cytoplasmic fibres are thrown at one end but the mitochondria and the Golgi elements remain uniformly scattered.
5. The cytoplasmic fibres are well preserved with Mann-Kopsch and chrome-osmium. They are destroyed to a large extent by fixatives containing acetic acid. Hence all the earlier workers do not figure them.
6. The "metaplasma" and the "formations ergastoplasmiques" of Blackman and Bouin brothers respectively are really the Golgi elements which are not completely destroyed by acetic acid.
7. During meiosis the mitochondria and the Golgi elements are distributed into two sub-equal parts.
8. When the spermatid is formed most of the Golgi rods assume the form of rings.
9. All the mitochondria and the Golgi elements go into the tail of the sperm and directly form the tail sheath.
10. The acrosome is probably secreted by a few Golgi rings which get stuck to the anterior face of the nuclear membrane of the maturing sperm. It is very prominent in the ripe sperm.
11. A few small granules constantly occur on that portion of the nuclear membrane of the maturing sperm from which the axial filament starts. They are intensely blackened with osmic acid and are very probably of the Golgi origin.

7. LIST OF LITERATURE.

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- (3) BOUIN, P. et BOUIN, M. *Bibliogr. Anat. Nancy*, **7**, 141.
- (4) MEDES, G. *Biol. Bull.* **9** and **10**, 156.
- (5) MEVES, F. und VON KORFF, K. *Arch. f. Mikr. Anat.* **57**, 481.
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- (7) PRENANT, A. *La cellule*, **3**, 415.

EXPLANATION OF PLATES.

Figs. 1, 5 and 15 have been magnified 780 times and the remainder 390 times.

FIG. 1. A spermatogonium showing mitochondria and Golgi rods (Benda).

FIG. 2. A spermatocyte showing mitochondria, Golgi rods and huge cytoplasmic fibres (Mann-Kopsch).

FIG. 3. A centrifuged spermatocyte. Mitochondria and the Golgi rods remain uniformly distributed but the cytoplasmic fibres have come to one end (Mann-Kopsch).

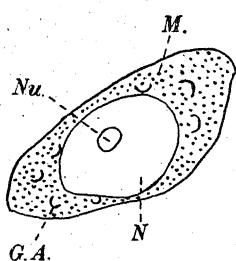


Fig. 1

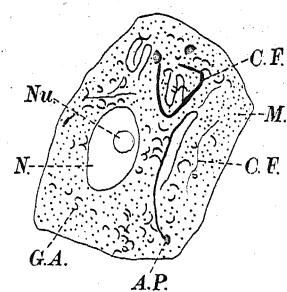


Fig. 2

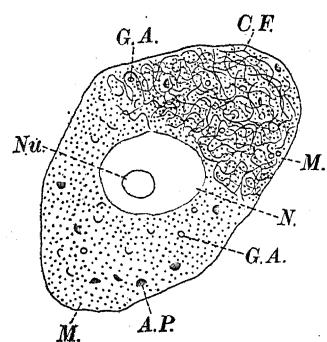


Fig. 3

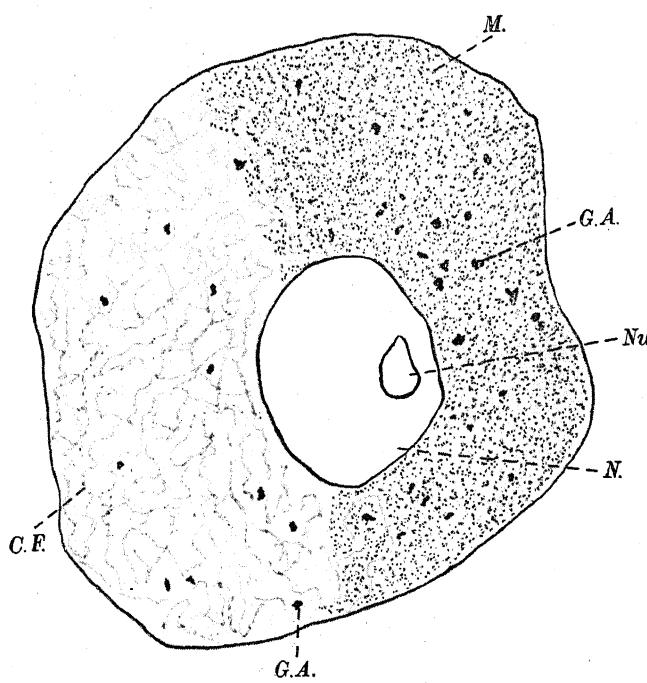


Fig. 5

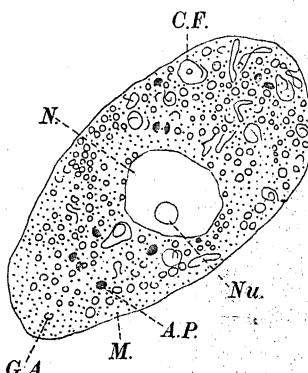


Fig. 4

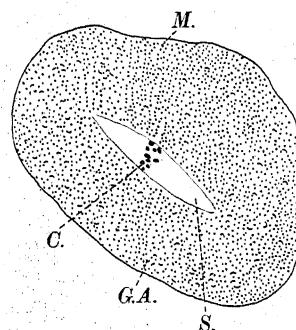


Fig. 6

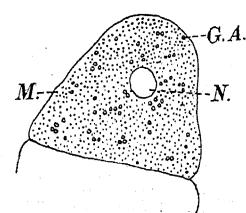


Fig. 7

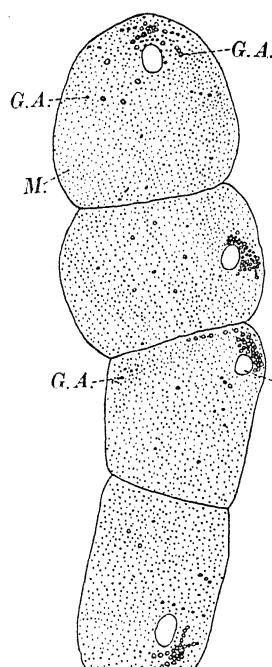


Fig. 8

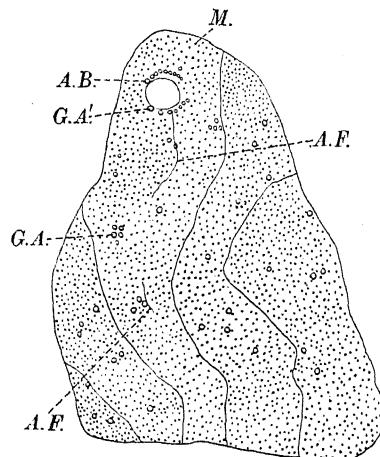


Fig. 9

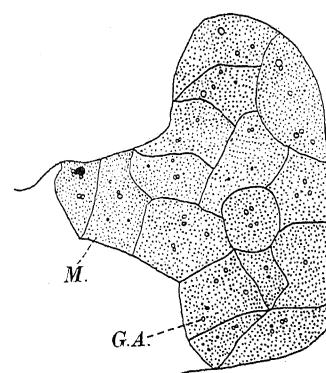


Fig. 10

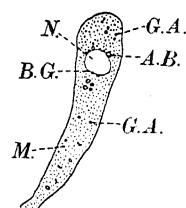


Fig. 11

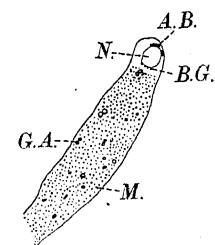


Fig. 12

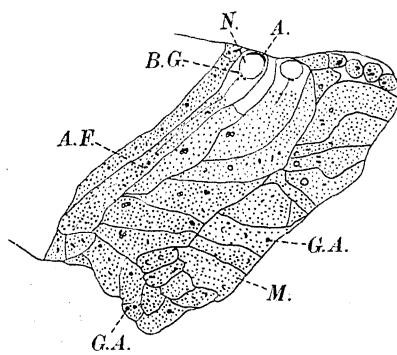


Fig. 13

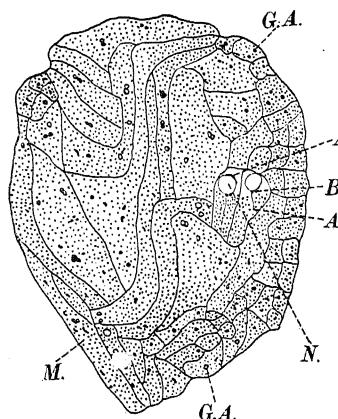


Fig. 14

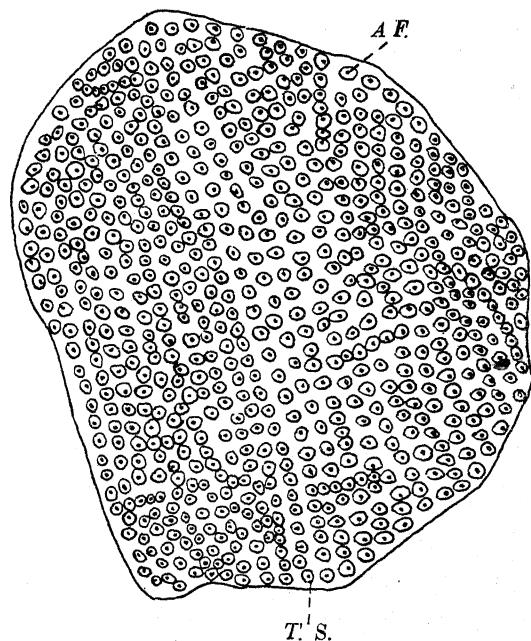


Fig. 15

FIG. 4. A centrifuged spermatocyte with extraordinary number of Golgi elements which hinder the movement of the cytoplasmic fibres to one end (Mann-Kopsch).

FIG. 5. A centrifuged spermatocyte (Gilson). Mitochondria and Golgi rods are distorted.

FIG. 6. Metaphase of second meiotic division. Mitochondria and Golgi elements are distributed in two sub-equal parts (Mann-Kopsch).

FIG. 7. A newly-formed spermatid. Most of the Golgi rods have become rings (Mann-Kopsch).

FIG. 8. Four spermatids. The nuclei have moved towards that part of the cell which will become anterior. Most of the Golgi elements lie in front of the nucleus (Mann-Kopsch).

FIGS. 9-14. Attenuating spermatids.

FIG. 9. Most of the Golgi elements have come into the tail. A few remain near the anterior face of the nucleus and are the acroblasts (A.B.). A few also remain at the posterior face of the nucleus (G.A.) and ultimately form the basal granules. Axial filament has appeared (Benda).

FIG. 10. Transverse section through the tail showing mitochondria and the Golgi elements (Mann-Kopsch).

FIG. 11. The acroblasts have become smaller and are stuck to the anterior face of the nucleus. The Golgi elements at the posterior face of the nucleus have also become smaller and have given rise to the basal granules (Mann-Kopsch).

FIG. 12. Basal granules are minute. Mitochondria have left the region immediately round the nucleus (Mann-Kopsch).

FIG. 13. Acrosome (A.) well defined. Basal granules (B.G.) at the root of the axial filament (Mann-Kopsch).

FIG. 14. Slightly older stage than that shown in Fig. 13.

FIG. 15. Transverse section through the tails of the ripe sperms showing axial filaments (A.F.) and tail sheaths (T.S.) (Mann-Kopsch).

REFERENCE LETTERS.

A. = Acrosome.

A.B. = Acroblasts.

A.P. = Archoplasm.

B.G. = Basal granules.

C. = Chromosomes.

C.F. = Cytoplasmic fibres.

G.A. = Golgi elements.

G.A. = Golgi elements which give rise to the basal granules.

M. = Mitochondria.

N. = Nucleus.

Nu. = Nucleolus.

S. = Spindle.

T.S. = Tail sheath.